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THE INFLUENCE OF THE GROWTH ENVIRONMENT ON THE
STABILITY OF A DRUG RESISTANCE PLASMID IN
ESCHERICHIA COLI K12 GROWN IN CHEMOSTAT CULTURE.

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A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy.

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April, 1980.

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ACKNOWLEDGEMENTS

I wish to thank Dr. J. H. Slater for his endless patience and the expert guidance and advice given to me throughout the course of this study.

I also wish to thank Professor D.P. Kelly and other members of the Department of Environmental Sciences for helpful discussions and I further gratefully acknowledge the expert assistance of the technical staff of this department. I also thank all the people cited in this thesis who provided me with bacterial strains and who introduced me to the techniques used in this study. Finally, I gratefully acknowledge the financial support of the Medical Research Council.

DECLARATION

I declare that this thesis is a report of the research undertaken by myself during the years 1976 - 1979 in the Department of Environmental Sciences under the Supervision of Dr. J. H. Slater. It is my own original, unaided work and has to the best of my knowledge not been previously described by any other persons.

SUMMARY

Populations of Escherichia coli K12 containing the plasmid TP120 which coded for resistance to ampicillin, streptomycin, sulphonamide and tetracycline were grown in continuous-culture under carbon-limited and phosphorus-limited conditions. The maintenance of the plasmid TP120 was studied and it was discovered that with time, resistance to one or more of the drugs was lost, resulting in the production of mutant populations which were more competitive than the parent population. The tetracycline resistance marker was always lost under carbon and phosphorus-limitations, but resistance to the other three drugs was lost only under phosphate-limited growth. A plasmid-minus strain was isolated on only one occasion. Strains of E. coli which had lost resistance to one or more drugs were shown to be smaller in size than the parent strain and were capable of higher maximum specific growth rates than the parent strain. Mixed culture competition experiments also demonstrated that evolved strains had lower K_s values than the original, parent strain. However the rate of carbon and phosphorus uptake measured in radiochemical experiments was unchanged for parent and evolved strains. The significance of plasmid stability within populations of bacteria in relation to the natural situation was also discussed.

ABBREVIATIONS

D	=	Dilution rate.
μ	=	Specific growth rate.
μ_{\max}	=	Maximum specific growth rate.
D_{crit}	=	Critical dilution rate.
S_R	=	Initial growth-limiting substrate concentration.
s	=	Final substrate concentration.
x	=	Biomass concentration.
Y	=	Growth yield.
K_s	=	Saturation constant.
MW	=	Molecular weight.
UV	=	Ultra violet.
Ap	=	Ampicillin.
Sm	=	Streptomycin.
Su	=	Sulphonamide.
Tc	=	Tetracycline.
Km	=	Karamycin.
Cm	=	Chloramphenicol.
Tp	=	Trimethoprim.

1. GENERAL INTRODUCTION

1.1. THE NATURE OF PLASMIDS

Plasmids, or extrachromosomal elements, are closed circular DNA molecules existing in bacteria, replicating independently of the chromosome and inherited by both daughter cells on cell division. Their existence has been known for the past twenty years and their genetics, epidemiology and biochemistry have been extensively investigated (Anderson, 1968; Chakrabarty, 1972; Clowes, 1972; Falkow, 1975; Helinski, 1973; Meynell, 1972; Bennett and Richmond, 1979). Plasmids were first identified in the Enterobacteriaceae but subsequently have been found in almost every bacterial group examined. Naturally isolated plasmids vary in size from the 2,250 nucleotide-long minicircular "cryptic" plasmids of Escherichia coli (Cozarelli, Kelly and Kornberg, 1968) to the large and complex F' plasmids which may contain more than 400,000 nucleotide pairs and carry up to 600 genes. Their diversity in size is also paralleled by wide variety of biological functions specified by plasmids and these are summarised in Table 1.1.

1.2. DRUG RESISTANCE PLASMIDS

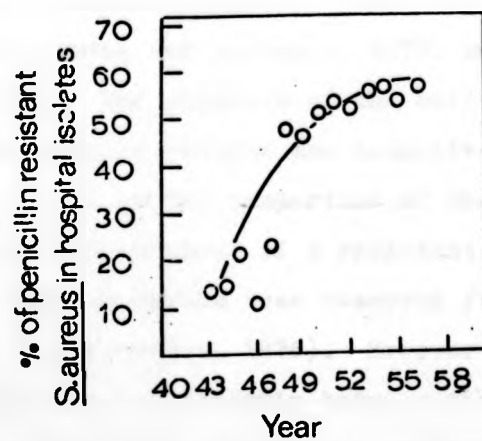
Most widely studied are the multiple drug resistance plasmids which were first discovered in Japan in 1950 (Watanabe, 1963) in strains of Shigella sp. during dysentery epidemics. There is now no doubt that antibiotic use favours the emergence of resistant bacterial populations (Anderson, 1968). This phenomenon has been observed on a world wide scale, where the introduction of a novel antibiotic is

Table 1.1. Examples of Plasmid-coded functions.

Biological function	Plasmid	Organism	Reference
Sex factors (fertility)	F	<u>Escherichia coli</u> .	Chandler and Krischnapallai (1974).
Antibiotic production	SCP 1	<u>Streptomyces coelicolor</u> .	Hopwood and Merrick (1977).
Enterotoxin production	Ent	<u>E. coli</u> .	Gyles, So and Falkow (1974).
Virulence factors-K88	Col, Hly.	<u>E. coli</u> .	Hardy (1975)
Colicin production	Col E 1, Col V	<u>E. coli</u> .	Schell and Montagu (1977).
Crown gall tumour	TI	<u>Agrobacterium tumefaciens</u> .	Gantotti, Patil and Mandel (1979).
Phaseotoxin production	pGP361	<u>Pseudomonas phaeoelocolor</u> .	Silver, Schottel and Weiss (1976); Clark, Weiss and Silver (1977).
Heavy metal resistance	R6, FP2	<u>E. coli</u> . <u>Pseudomonas aeruginosa</u> .	Grindley, Humphreys and Anderson (1973)
Antibiotic resistance	RI, R6, RP4.	<u>E. coli</u> and others.	Molina, Banduri, Tamaro, Venturri and Monti-Bragadini (1979)
U.V. resistance	R46, Col 1b	<u>E. coli</u> .	Tweats, Thompson, Pinney and Smith (1976)
Metabolism of Toluene, Camphor, xylene etc.	Tol, Cam, Xyl.	<u>Pseudomonas putida</u> .	Chakrabarty (1976) Williams and Murray (1974)

frequently followed by the appearance of bacteria resistant to that agent (Figure 1.1) (Munch, Peterson and Boundy, 1972; Acar, Bouanchand and Chabbert, 1977). Another example was the introduction of carbenicillin into the Burns Unit at a hospital in Birmingham, England in 1966 for the treatment of infections involving Pseudomonas aeruginosa. In March 1968 a strong penicillinase activity was detected in isolates of P. aeruginosa and this capability was found to be specified by a bacterial plasmid present in different strains (Lowbury, Kidson, Lilley, Aycliffe and Jones, 1969). This plasmid, shown to belong to the P incompatibility group, coded for resistance to tetracycline, ampicillin and novobiocin and had a molecular weight of 40×10^6 . It continued to survive in bacterial hosts for 3 years at which point the same plasmid was found in Klebsiella aerogenes as well as P. aeruginosa (Sykes and Richmond, 1970) even though carbenicillin had not been administered during that period. However, the fear that all organisms capable of drug resistance factor transfer might become resistant to all antibiotics has not been realised. In fact, there is some evidence that the incidence of antibiotic resistant organisms may have levelled off, or even fallen, for at least some organisms found in the human population (Slocombe and Sutherland, 1972; Bulger, Larson and Sherris, 1970). Consequently because of the importance of plasmid-borne resistance mechanisms for the health of human populations, the stability of antibiotic resistance plasmids and their ability to survive and transfer their

Figure 1.1. The incidence of penicillinase producing Staphylococcus aureus in hospital infections in the years 1943 - 1957 (redrawn from Richmond, 1977).



resistance have received considerable attention. The behaviour of plasmids and plasmid carrying organisms has been investigated using in vivo and in vitro experimental techniques.

1.3. SURVIVAL OF PLASMIDS IN VIVO

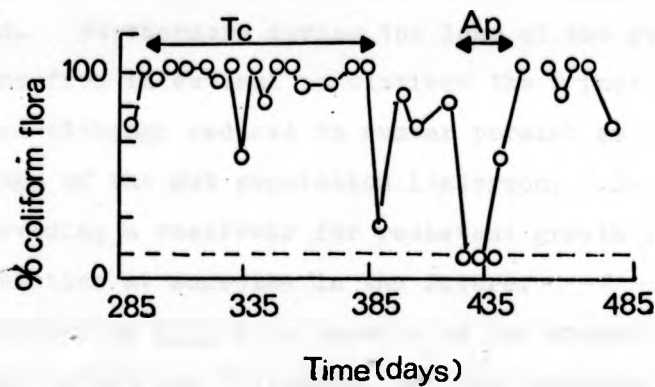
The use of drugs favours the selection of resistant populations such that a therapeutic course of an antibiotic commonly results in the conversion of the bacterial population in a human alimentary tract to a resistant state (Anderson, Gillespie and Richmond, 1973; Smith, 1971; Richmond, 1975). The presence of the antibiotic encourages resistant bacteria to outgrow the sensitive organisms with a resulting change in the properties of the whole population.

The long term survival of a resistant E. coli strain in an individual human being has been observed for periods of up to 9 months (Petrocheilou, 1976). However there is a slightly ambiguous relationship between the persistence of resistant E. coli strains and their antibiotic resistance mechanisms. This was illustrated in a survey which reported the incidence of tetracycline resistant E. coli in one person following the termination of a prolonged period of tetracycline therapy (Petrocheilou, Richmond and Grinsted, 1977 & 1979). Antibiotic use led to the establishment of a tetracycline-resistant E. coli as the majority component of the faecal flora (Figure 1.2). After 385 days from the start of the survey, tetracycline therapy was stopped and not resumed. The resistant E. coli persisted within the flora for several months in the absence of tetracycline selection. On day

245 ampicillin treatment was administered for 10 days and during this period the tetracycline-resistant organisms were undetectable, although ampicillin-resistant E. coli were abundant (Figure 1.2.). Immediately after ampicillin treatment ended the ampicillin-resistant strain were replaced by the tetracycline-resistant E. coli. More routinely, R factor-bearing organisms have been shown to decline in gut populations approximately 4 days following ingestion (Anderson, J.D. 1974; Lacey 1975). This is usually due to the successful growth of the sensitive derivatives in the absence of the drug selection pressure, resulting in the competitive exclusion of the resistant strain. A variety of factors have been suggested as influencing the survival of the plasmid-carrying organisms, including: the concentration of competing organisms; the presence of bile salts; and inert bacterial matter, since these factors are all thought to inhibit plasmid transfer (Anderson, J.D., 1975); Anderson, J.D., (1974) also suggested that the plasmid-bearing bacteria had an impaired "vitality" compared to the sensitive organisms.

These experiments demonstrate considerable variation in the stability of the plasmids examined. Hartley et al. (1975) suggested that the outcome depended more often on the colonising properties of the host strain rather than on the plasmid present in a particular host. Thus the displacement of resistant E. coli by sensitive strains is due to the fact that R plasmids are not in general carried by those E. coli

Figure 1.2. The effect of the termination of tetracycline therapy on the persistence of an E.coli strain 075 Tc in a single individual. (redrawn from Richmond, 1977).



strains which are effective at becoming dominant components of the faecal flora (Hartley, Howe, Linton and Linton, 1975). This property may account for the observation that the majority of human subjects carry only a small proportion of resistant E. coli in their alimentary tract at all times (Datta, 1969), although resistant strains which account for more than 10% of the faecal flora in some humans have been reported. Furthermore during the loss of the resistant organisms from intestinal populations the R factor-carrying organisms although reduced in number persist as a very small percentage of the gut population (Anderson, J.D., 1974), thus providing a reservoir for resistant growth if challenged by antibiotics at sometime in the future.

Survival in vivo also depends on the animal host since in animal intestines R plasmids are more commonly carried by E. coli strains which are also good colonisers (Smith, H.W., 1975). The transfer and survival of resistant bacteria in animal and poultry hosts has been described by many authors (Smith, M.G., 1975, 1977a, 1977b) and even their subsequent transfer and colonisation of the human gut reported (Linton, Howe, Bennet, Richmond and Whiteside, 1977).

Indirect evidence provided by the isolation of resistant organisms from natural sources also suggests that in water supplies and raw sewage, drug resistant coliforms show a long term survival and capacity to transfer resistance factors in mixed populations (Grabow, Prozesky and Smith, 1974); Smith, H.W., 1970; Freary, Sturtevant and Lawford, 1972; Hughes and Meynell, 1975).

1.4. SURVIVAL OF PLASMIDS IN VITRO

The first group to consider the survival of R factor carrying E. coli in mixed cultures using continuous-flow culture (Melling, Ellwood and Robinson, 1977) reported the prolonged persistence of the plasmid RPl within populations at a range of growth rates from $0.05 - 1.0 \text{ h}^{-1}$ grown under phosphorus, carbon and magnesium-limited conditions. The plasmid stability was investigated further by the examinations of plasmid-carrying (R^+) strains growing in direct competition with plasmid-free (R^-) strains in the same nutrient limitations. The R^- strain was only successful in phosphorus-limited conditions resulting in a reduction of the number of R^+ organisms at several dilution rates. The rate of loss of the resistant organisms approximated to the theoretical washout rates (Section 1.7.6.) but did not result in complete removal of R^+ organisms. Instead the resistant population was maintained at a level of approximately 1-5% of the total population. In conditions of carbon and magnesium limitation, the outcome of competition depended on the ratio of resistant to sensitive organisms at the onset of competition and that for a ratio above 1:1 the organism in excess was always successful.

In a more recent study (Wouters, Rops and van Andel, 1978; Wouters and van Andel, 1979) the persistence of an E. coli carrying the plasmid R6 at high and low growth rates under carbon, nitrogen or phosphorus-limitation, and in anaerobic conditions was briefly described. The period of

persistence of the plasmid was not stated but in competition experiments between isogenic R^- and R^+ strains the plasmid-minus strains were successful in all conditions examined for periods of up to 100 h.

Adams, Kinney, Thompson, Rubin and Helling (1979) conducted a similar study using a laboratory constructed plasmid. The plasmid RSF 2124 was a hybrid colicin-producing plasmid with the transposon Tna conferring ampicillin resistance inserted in it. Other plasmids examined contained in addition DNA cloned from a chloroplast of Euglena gracilis. This comprehensive study provided a valuable insight into plasmid behaviour and made an interesting comparison with the N group plasmid TPl20., the subject of this thesis. The success of the plasmid-minus or plasmid-carrying organisms depended on the starting ratio of the two populations. This frequency-dependent effect was due to the colicin producing ability of the plasmid-carrying strain. The Col^+ strain was lost routinely from cultures where it had a starting frequency below 50% of the population. This loss was due not to the loss of the plasmid from host organisms during segregation but was caused by the slower growth rate of the Col^+ organisms compared to the plasmid-free strain. Strains carrying different plasmids were lost at variable rates. This was considered to be a function of the properties coded for by the particular gene in the cloned DNA segment and its influence on the host organism and not just simply due to the increased size of the plasmid. From

these observations Adams et. al. (1979) concluded that whilst the maintenance of the plasmid within the population was due to selection pressures provided by colicins or antibiotics, other mechanisms of maintenance were also operating although these were not so obviously selected for. Insertion sequences in the plasmid could provide sites for recombination and hence increase the genetic lability of the plasmid and facilitate addition or duplication of bacterial genes. This means that plasmid-carrying organisms would be selected for in the same way as mutator genes in chemostat culture (Gibson, Scheppe and Cox, 1972; Painter, 1975). Therefore, theoretically, if following the reduction of the Col⁺ strain to a low percentage of the total population during continuous-flow culture, the experiment was continued for a sufficiently long period the plasmid-carrying organisms would eventually replace the plasmid-free organisms even in the absence of colicin selection. The selectively favoured cells should possess an altered structure reflecting the acquisition of, for example, rate - limiting genes for growth on low levels of glucose.

Jones and Primrose (1979) have similarly studied the survival of laboratory constructed plasmid chimerae and demonstrated the loss of the tetracycline resistance capacity occurring swiftly during growth in nutrient-limited conditions.

Dale and Smith (1979), instead of culturing plasmid-carrying organisms in chemostat culture, continuously subcultured the R⁺ strains in batch culture. Organisms bearing the constructed non-conjugative plasmids pH 121 or

pB 165 were found to have slower growth rates compared to plasmid-minus isogenic strains in mixed cultures. Consequently the plasmid containing population declined to only 0.1% of the total population at the end of 6 subcultures. The ability of the organisms to survive extended incubation was also tested by incubating pure and mixed cultures of resistant and sensitive E. coli 114 at 37° C for approximately 10 months. In pure culture, resistant and sensitive strains behaved in similar ways, whilst in mixed culture, the proportion of streptomycin resistant organisms declined exponentially such that after 100 days it was no longer possible to isolate resistant organisms from the culture.

In a similar, repeated subculture experiment involving naturally isolated R plasmids of gonococci Falkow, Elwell, Roberts, Heffron and Gill (1977) described the loss of a small plasmid with a molecular weight of 4.4×10^6 from a clinical isolate. The plasmid carrying cells were reduced to frequencies ranging from 0.1% to 70% of the total population, which indicated that again a reservoir of resistant organisms remained in the population. Falkow et al. (1977) also noted that in the absence of the antibiotic selection pressure there was a tendency for the gonococci hosts to lose their plasmids at a higher frequency than R plasmid-carrying enteric strains.

Thus, the results of studies of the stability of plasmids within bacterial populations in in vitro conditions indicate that, as in the in vivo situation, the survival of the plasmid

depended on two important parameters, namely:

- (1) the nature of the host organisms and
- (2) the effect of the plasmid on the host organism, particularly by causing a less efficient and slower growth rate than the plasmid-minus isogenic strain.

1.5. CLOSED-CULTURE GROWTH OF PLASMID-CARRYING ORGANISMS

A number of closed-culture studies have shown that plasmid-carrying organisms have lower maximum specific growth rates than plasmid-minus strains grown in the same conditions (Terawaki, Kakizawa, Takayasu and Yoshikawa, 1968; Yokota, Kasuga, Kaieko and Kuwahara, 1972; Shaeffler 1974, 1972; Grinsted and Lacey, 1973; Lacey, Lewis and Rosdahl, 1975; Hershfield et al., 1975; Lacey, 1975; Kayama and Yara, 1975; Inselburg, 1978; Nakazawa, 1978). The host organisms examined have ranged from strains of E. coli (e.g. Kayama and Yara, 1975; Inselburg, 1978) to Staphylococci as described by Lacey (1975) and also Vibrio cholerae (Yokota et al. 1972). Watanabe D.S.A. (1978) demonstrated that mutations to multiple-drug resistance in a plasmid carried by an E. coli host interfered with both the lag phase and exponential growth rate of the host strain. Triple mutations were more compromising than double which, in turn, were more detrimental than a single mutation when compared to the original sensitive strain. This evidence has suggested that, in drug-free media the resistance functions of the plasmids were redundant and since synthesis and replication of the plasmid and also synthesis of its coded properties utilised

elemental and energy resources which otherwise would be diverted into biomass production and an increased growth rate, the plasmids could be dispensed with.

Zamenhof and Eichorn (1976) showed that amino acid auxotrophs of Bacillus subtilis had marked growth rate advantages over isogenic prototrophic strains in carbon-limited growth conditions. Furthermore the greater the number of biosynthetic sequences dispensed with by the auxotroph the greater was the growth rate advantage over the prototroph. Conversely organisms which wasted growth nutrients, for example by the over production and excretion of an amino acid, were at a growth disadvantage (Zamenhof and Eichorn, 1967; Baich and Johnson, 1968). Lacey, Lewis and Rosdahl (1974) reported a change of penicillinase production from macroconstitutive to microconstitutive production during an in vivo evolution study of Staphylococcus aureus. Strains which produced the enzyme macroconstitutively had longer doubling time (30 min.) than organisms which either produced the penicillinase microconstitutively (25 min.) or those which did not produce the enzyme at all (24 min.). In mixed culture the microconstitutive enzyme producing organisms outgrew the macroconstitutive strain within 20 h.

Also plasmid cloning vehicles, derived from plasmids under relaxed control which have a high copy number during normal growth and therefore can be used to produce high yields of gene products, were rapidly lost from populations during growth in the absence of selection pressure, due to a significantly reduced growth rate (Hershtfield, 1974).

Conversely some authors have reported no change in the growth rates of bacterial strains which have received plasmids by conjugation (Søgaard, 1975; Cullum, Collins and Broda, 1978b, Nordstrom, 1977). Other reports have indicated that the differences in growth rates were not detectable in pure culture but became apparent in mixed cultures grown in batch or chemostat systems (Dale and Smith, 1979; Adams et al., 1979). Dale and Smith (1979) stated that while there was no difference in the exponential growth rate of cultures of plasmid-carrying or plasmid-free organisms, there was, however, a slower growth phase in plasmid-minus populations immediately prior to the stationary phase. This slower phase was absent in the plasmid-carrying populations which went rapidly into the stationary growth phase from the exponential growth phase.

1.6. PLASMID EVOLUTION

In vivo and in vitro examination of markers borne on drug resistant plasmids have led to suggestions about the mechanisms of plasmid evolution. Early steps of plasmid evolution probably involved the assembly of deoxyribonucleotides to form a site for the initiation of DNA replication (a replication origin) and acquisition of genes that specified functions required for autonomous replication (Cohen, 1976). Subsequent linkage of the resulting replication segment to one or more genes that provided a biological advantage to cells carrying the plasmid (for example drug resistance

markers) would then aid its propagation. Later steps in the evolution of some plasmids might then involve the addition of groups of genes that facilitate inter-bacterial transfer or an extra-cellular existence. The mechanisms involved in the "build-up" of the structural composition of a plasmid are thought to be the same as those involved in the dissociation or loss of markers from plasmids. In 1978 Timmis et al. noted that various changes (both loss and gain of DNA) in the conjugative plasmid R6-5 had occurred since its isolation from a single clone in 1971. Using R6-5 as a model they were able to make observations about the process of plasmid evolution and to draw general conclusions about plasmid evolution. They suggested that plasmids isolated from natural sources had evolved over many bacterial generations as a result of in vivo recombination events that produced DNA sequence arrangements which were optimal for the functioning and propagation of plasmids and were therefore unlikely to undergo major changes unless subjected to additional selection pressures. On the other hand, those plasmids which had been newly constructed in vitro (as those studied by Jones and Primrose 1979; Adams et al., 1979) may have DNA arrangements either that are less favourable for optimal functioning of certain genetic regions or that are more susceptible to attack by the cellular recombination system. In either case these constructed plasmids could be subject to extensive structural rearrangements in order to reach a stable basal state.

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Cohen (1978) reported that changes in plasmid structure occurred by two methods, both due to site-specific illegitimate recombination, that is, independent of the host cells *rec A* gene product. The first, termed macroevolution, led to the insertion, deletion or rearrangement of relatively large segments of DNA and the second, termed microevolution, involved such processes as insertion, deletion or substitution of very short DNA segments.

Macroevolution of plasmid DNA can be resolved by heteroduplex analysis which is capable of revealing changes in DNA sequences greater than 50 - 70 base pairs. Such recombination events have been shown to involve insertion sequences (IS) and transposable elements (Tn) both of which are not only capable of undergoing translocation to new sites on a genome but also promote, in their vicinity, other site-specific recombination events such as deletions and substitutions. Transposons having a MW between 2×10^6 - 15×10^6 (1.2) have been extensively reviewed (Cohen, 1976; Kleckner, Roth and Botstein, 1977; Kleckner, 1977; Nevers and Saedler, 1977) and many of the drug resistance genes in bacteria exist as part of specialised segments of DNA. Heteroduplex analysis has revealed that many of the transposon elements have an unusual structure in that the two ends of the transposon DNA consist of stretches of homologous base pairs. These stretches of homology can be orientated in the same direction with respect to the intervening sequences (a direct repeat as found in Tn5 and Tn10 (Table 1.2)) or as an inverted repeat (found in Tn1, Tn2, Tn3 and Tn4 (Table 1.2)). The structure is shown

Table 1.2. Transposons. ^aAp = Ampicillin; Sm = Streptomycin; Su = Sulphonamide; Km Kanamycin
Tnp = Trimethoprim Cm = Chloramphenicol; Tc = Tetracycline.

Transposon	Associated drug resistance ^a	Size (x10 ⁶ daltons)	Terminal repetitions	Polarity	Deletion generation	Plasmid origin	Reference
Tn 1	Ap	3.2	140bp.inv.	Yes	Yes	RP4	Hedges and Jacob (1974).
Tn 2	Ap	3.2	140bp.inv.	Yes	-	RSF1030	Heffron et al. (1975a)
Tn 3	Ap	3.2	140bp.inv	-	-	R1.	Kopecko and Cohen (1975)
Tn 4	Ap, Sm, Su.	13	140bp.inv (Tn 3)	-	-	R1.	Kopecko and Cohen (1975).
Tn 5	Km.	3.5	1460 bp.inv. Yes	Yes	Yes	JR67	Berg et al. (1975)
Tn 6	Km	2.7	? direct	-	-	JR72	Berg et al. (1975)
Tn 7	Tp Sm	9	?	-	-	R483	Barth et al. (1975)
Tn 9	Cm	1.7	800bp. direct	Yes	Yes	PSM14	Gottesman and Rosner (1975).
Tn 10	Tc	5.5	R100	Yes	Yes	R100	Foster et al. (1975) Kleckner et al (1975).

in Figure 3. The mechanism of the transposition of these elements from replicon to replicon and the processes of excision and insertion has not been elucidated. The process may involve the homologous base pair sequences or it has been suggested that the transposon may form loop structures (Figure 1.4.2.) similar to the Campbell model for the integration of phage λ into the E. coli chromosome. Although the actual configuration during recombination is unknown, it seems probable that there are site specific enzymes capable of recognising the inverted repeat termini of the elements involved. Furthermore, insertion of the transposons into a replicon is not random but instead clusters within certain fragments of the DNA sequence so that there is a regional specificity involved (Cohen, 1976). Datta, Hughes, Nugent and Richards (1979) followed the stability and mutability of a transposon (possibly Tn7 (Table 1.2.)) present on a plasmid during an hospital infection. This transposable element underwent insertion into the host chromosome and while generally no structural variations in the transposon (SmTp) was observed in a single event the trimethoprim marker was transposed without streptomycin resistance presumably as a result of deletion of the DNA which the authors were unable to detect physically. Other transposons particularly those bearing resistance to ampicillins, for example Tn1, Tn2 have been isolated from many different plasmids in bacteria of many genera (Laufs and Kaupers, 1977) and have a world wide distribution (Campbell, Berg, Lederberg, Starlinger, Botstein, Novick and Sybalski, 1977). Transposons, therefore, provide a ready vehicle for

Figure 1.3. Diagrammatic representation of the structure of a tetracycline resistant transposon showing inverted repeat structure (1) in a heteroduplex DNA molecule (2) in a homoduplex DNA molecule. (redrawn from Bennet and Richmond, 1978)

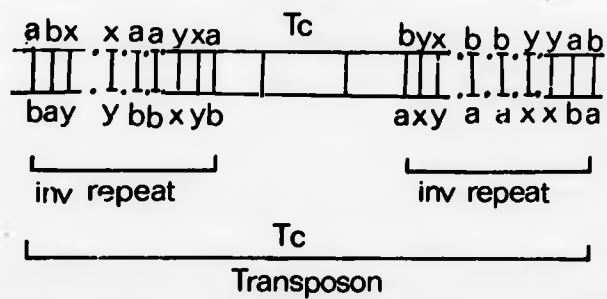
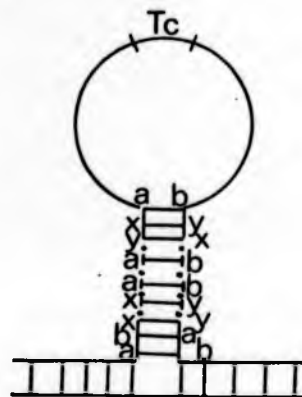
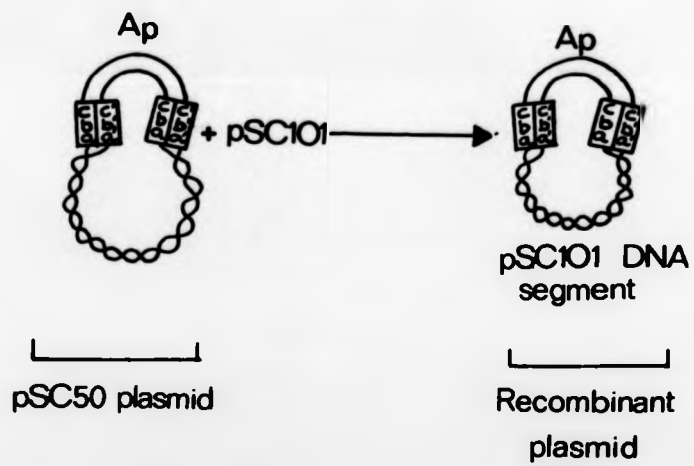
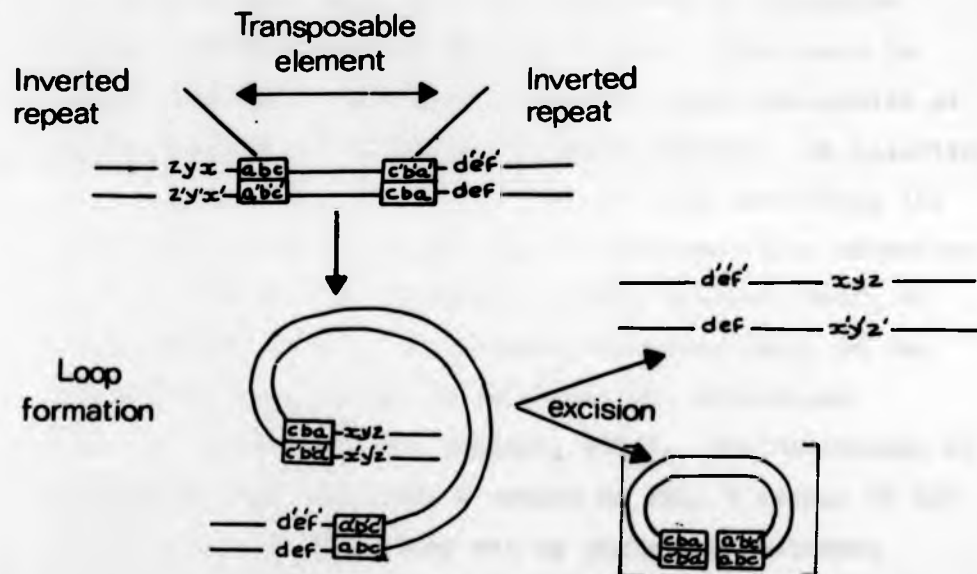


Figure 1.4 Diagrammatic representation of translocation of Ampicillin resistant transposon (Tna) from plasmid (pSC50) to new recombinant plasmid (pSC101). (1) Translocation involving the ends of the inverted repeat sequence. (2) Possible Campbell model for the configuration of the DNA segment involved in translocation. The separated Tna segment is shown in brackets since its existence as a separate unit has not been demonstrated (redrawn from Cohen, 1976).

(1)



(2)



the acquisition or removal of markers from a plasmid structure.

Insertion sequences, on the other hand, do not code for resistance functions or indeed any functions other than those concerned with insertion. Their structure and role in recombination have been the subject of an extensive review by Starlinger and Saedler (1976). They range in length from 800 - 1400 base pairs and become integrated at multiple sites on bacterial and phage genomes. On insertion into a genome they are capable of not only abolishing the expression of the gene but also of exerting polar mutations (Jordon, Saedler and Starlinger, 1968; Saedler, Reif, Hu and Davidson, 1974). IS elements have been found in the chromosome and plasmids of bacteria (Hu, Ohtsubo and Davidson, 1975; Chow and Bukhari, 1976). The chromosome of E. coli has approximately 8 copies of IS1, 5 copies of IS2 and 3 copies of IS3. They set up regions of sequence homology in replicons which can subsequently be used for gene exchange in classical recombination (Saedler and Heiss, 1973; Hu et al., 1975). The reversible dissociation of cointegrate plasmids into a resistance (r)-determined segment and resistance transfer region (RTF) occurs at the site of IS1 elements on these plasmids (Cohen, 1976). IS sequences are also present as the repeated sequences at the ends of some transposons, for example, the ends of Tn9 are in fact homologous to the insertion sequence IS1 (Ptashne and Cohen, 1975; MacHattie and Jablowski, 1977).

The second mechanism of plasmid evolution, that is microevolution, can only be detected by a change in

Table 1.3. Insertion Sequences.

Insertion Sequence	Size (base pairs)	Polarity	Deletion generation
IS1	800	Yes	Yes
IS2	1350	Yes	Yes
IS3	1400	Yes	-
IS4	1400	Yes	-

endonuclease cleavage pattern of the plasmid during growth of the organism or following transformation (Chang, Lassman, Clayton and Cohen, 1975; Cohen, Cabello, Casaban, Chang and Timmis, 1978) as often observed during the cloning of hybrid plasmids. It presumably involves such processes as insertion, deletion or substitution of very short segments of DNA. Cohen et. al., (1978) reported that the extent of microevolution in the R6-5 plasmid and in its constructed hybrids was surprisingly great since no intentional selection for microevolutionary changes had been exerted. He concluded that such spontaneous DNA sequence alteration reflected a rate of evolutionary change that was hitherto unsuspected.

Therefore this study aimed to investigate any possible changes or evolution in plasmid structure occurring in the controlled conditions of nutrient limitation and fixed growth rates of the chemostat.

1.7. THE THEORY OF CONTINUOUS-FLOW CULTURE.

The growth of microorganisms in continuous-flow culture has many advantages for observing the process of plasmid evolution within a bacterial population.

Chemostat culture systems provide highly selective growth environments in which to examine the ability of microorganisms to compete or coexist (Novick and Szilard, 1950; Harder, Kuenen and Matin, 1977; Slater, 1979). The advantages of examining the behaviour of the plasmid-carrying organisms in open rather than closed culture can be summarised as follows:-

- (1) Chemostat culture studies are more reproducible compared

to batch growth cultures.

(2) Chemostat culture enables the growth rate to be varied whilst maintaining a constant environment (or vice versa).

Thus continuous-flow systems allow for a more rigorous examination of the effects of individual environmental factors on the physiology of microorganisms and, in this case, their effect on plasmid stability and evolution. The behaviour of different organisms can also be compared.

(3) The growth can be controlled and maintained at a pre-determined value for long periods, enabling investigation of many generations of microorganisms and enabling the establishment of steady state conditions.

(4) Substrate-limited growth conditions can be established and this permits the study of microbial physiology under different limitations. In many cases substrate-limited conditions mimic those which occur in nature (Brown, 1977).

(5) It enables the investigation of mixed populations for a wide variety of relationships (Bungay and Bungay, 1968; Veldkamp and Jannasch, 1972; Meers, 1973) and produces more reliable results than batch system investigation of mixed populations. Since Powell (1958) first discussed the growth of contaminants and mutants in continuous-flow culture at fixed dilution rates, the use of the chemostat in the study of mixed populations has been extensively reviewed (Fredrickson, 1977; Taylor and William, 1975; Tempest, Dicks and Meers, 1976; Slater and Bull, 1978).

1.7.1. The kinetics of continuous-flow culture growth.

Consider a culture vessel containing a fixed volume

of culture, V , which is vigorously stirred to ensure a homogeneous cell suspension. A defined growth medium containing a single growth-limiting substrate at an initial growth-limiting substrate concentration, S_R , is pumped into the vessel at a constant flow rate, F . Within the culture vessel the incoming fresh medium is instantaneously mixed into the culture and some used to give a biomass concentration, x . As a result of growth, the growth-limiting substrate is reduced to concentration, s . In order to maintain a fixed volume some of the growing culture is removed from the growth vessel at the same rate as fresh medium is introduced into the growth vessel.

1.7.2. The dilution rate.

The specific growth rate of the organism depends on the concentration of the limiting nutrient (Monod, 1942). In chemostat culture the concentration of the limiting substrate depends on the rate at which fresh substrate is supplied, that is, on the dilution factor as the substrate is dispersed throughout the culture vessel and on the rate of removal of unused growth-limiting substrates. Thus, the growth-limiting substrate concentration depends on the ratio of the flow rate, F , and the culture volume, V , a parameter known as the dilution rate, D ;

$$D = \frac{F}{V} \quad (1.1)$$

The dilution rate has units of reciprocal time, usually h^{-1} , and is a measure of the number or fraction of the culture volume changes achieved in unit time.

1.7.3. The dilution rate and biomass concentration.

Within the chemostat culture there is a biomass balance which depends on the rate of biomass production due to growth minus the rate of biomass removal by washout. Thus:

$$\frac{dx}{dt} = \mu x - Dx \quad (1.2)$$

and so:
$$\frac{dx}{dt} = x (\mu - D) \quad (1.3)$$

substituting for μ by equation 1.5 we have:

$$\frac{dx}{dt} = x \left[\frac{\mu_{\max} s}{(K_s + s)} - D \right] \quad (1.4)$$

where μ_{\max} is the maximum specific growth rate; s is the concentration of the growth-limiting substrate and K_s is the saturation constant, numerically equal to the substrate concentration which gives $\mu = \frac{1}{2} \mu_{\max}$

With reference to equation 1.2 there are three different situations which need to be considered:-

- (1) If $\mu > D$, then dx/dt is positive the biomass concentration in the culture vessel increases since the rate of biomass production exceeds the rate of biomass washout.
- (2) If $\mu < D$ then dx/dt is negative and the biomass concentration decreases since the rate of culture washout is greater than the rate of biomass production.
- (3) If $\mu = D$, then $dx/dt = 0$ and the biomass concentration remains constant and the culture is said to be in a steady state.

1.7.4. The dilution rate and growth limiting substrate concentration.

Monod (1942) first established that the specific growth rate, μ , depends upon the growth-limiting substrate concentration

in a closed culture system according to the equation:

$$\mu = \mu_{\max} \left[\frac{s}{K_s + s} \right] \quad (1.5)$$

where μ is the specific growth rate; μ_{\max} is the maximum specific growth rate; s is the concentration of the growth-limiting substrate and K_s is the saturation constant numerically equal to the substrate concentration which gives $\mu = \frac{1}{2} \mu_{\max}$

A similar growth-limiting substrate balance equation can be formulated for continuous-flow culture. The rate of change of growth-limiting substrate concentration in the culture vessel depends on the rate of input of fresh substrate; the rate of unused substrate removal (washout) and the rate of substrate utilisation by the organism. Thus:

$$\frac{ds}{dt} = DS_R - Ds - \frac{\mu x}{Y} \quad (1.6)$$

$$\text{i.e. } \frac{ds}{dt} = D(S_R - s) - \frac{\mu x}{Y} \quad (1.7)$$

where the final term is substituted from equation (1.10); D is the dilution rate; S_R the initial growth-limiting substrate concentration; s the final substrate concentration; x is the biomass concentration and Y the observed growth yield which is defined as the quantity of biomass produced in unit time as the result of utilisation of unit amount of the limiting nutrient in the same time. Thus for a growing culture in a small time interval

$$\frac{dx}{ds} = Y \quad (1.8)$$

$$\text{and since } \frac{dx}{dt} = \mu x \quad (1.9)$$

$$\text{then } -\frac{ds}{dt} = \frac{\mu x}{Y} \quad (1.10)$$

As in the case of the rate of change of biomass concentration there are three possible situations which need to be considered.

(1) If $\mu > D$ then ds/dt is negative and the growth-limiting substrate concentration decreases. The biomass concentration is increasing thereby resulting in a decrease in growth-limiting substrate concentration.

(2) If $\mu < D$ then ds/dt is positive and the growth-limiting substrate concentration increases. Thereby the biomass concentration decreases resulting in the increase in growth-limiting substrate concentration.

(3) Finally if $\mu = D$ then $ds/dt = 0$ and the growth limiting substrate concentration reaches a constant, steady state value at the same time as the biomass concentration reaches a steady state.

1.7.5. The steady-state.

Thus from equation 1.4 and 1.7

$$0 = \bar{x} \left[\frac{\mu_{\max} \bar{s}}{K_s + \bar{s}} + D \right] \quad (1.11)$$

where \bar{s} and \bar{x} indicate the steady state growth growth-limiting substrate and biomass concentrations, respectively

Thus:

$$D = \frac{\mu_{\max} \bar{s}}{K_s + \bar{s}}$$

and

$$\bar{s} = \frac{DK_s}{(\mu_{\max} - D)} \quad (1.12)$$

Also:

$$0 = D (S_R - \bar{s}) - \frac{\mu \bar{x}}{Y}$$

and

$$\bar{x} = Y (S_R - \bar{s}) \quad (1.13)$$

Equations 1.12 and 1.13 enable the steady-state concentrations at any dilution rates to be predicted, provided that the initial growth-limiting substrate concentration and the three growth parameters, namely μ_{\max} , K_s and Y are known.

1.7.6. The critical dilution rate and the calculation of maximum specific growth rate from culture washout kinetics .

From equation 1.3 it can be seen that there is an upper dilution rate above which steady-state cultures cannot be established. This is because the organism's specific growth rate has a maximum value, μ_{\max} , which is genetically determined and therefore cannot be exceeded for a given set of growth conditions. Thus if $D > \mu_{\max}$, then $\frac{dx}{dt}$ must be negative, and a steady-state cannot be obtained and the culture is said to washout. There is a unique dilution rate known as the critical dilution rate, D_{crit} , fractionally below which steady-state cultures are theoretically possible and fractionally above which culture washout occurs. In fact D_{crit} is reached when $\bar{s} = S_R$ and so from equation 1.12, substituting from \bar{s} and D and rearranging, we have:

$$D_{\text{crit}} = \frac{\mu_{\max} \cdot S_R}{S_R + K_s} \quad (1.14)$$

Therefore D_{crit} is a good approximation to the maximum specific growth rate under the prevailing conditions provided that the concentration of the growth-limiting substrate in the inflowing medium, S_R , is considerably greater than the organisms saturation constant for the growth-limiting nutrient.

During washout from a culture vessel when $D > \mu_{\max}$ the

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organisms grow at μ_{\max} provided that $s \gg K_s$ and so the kinetics of washout may be used to calculate μ_{\max} values since:

$$\frac{dx}{dt} = x (\mu_{\max} - D)$$

where D is a known value greater than μ_{\max} .

This has the solution:

$$x_t = x_0 e^{(\mu_{\max} - D)t}$$

where x_0 is the organisms concentration in a steady-state at time $t = 0$. When the dilution rate is increased stepwise to a value where $D > \mu_{\max}$

$$\text{Thus:} \quad \ln x_t = \ln x_0 + (\mu_{\max} - D)t$$

$$\text{and} \quad \mu_{\max} = \frac{(\ln x_t - \ln x_0)}{t} + D \quad (1.15)$$

1.7.7. The kinetic principles of competition between microbial populations.

Competition is said to occur when two (or more) populations growing in the same habitat or niche are limited either in terms of their specific growth rate or final population size, as the result of a common dependence on an external factor required for growth (Gause, 1934; Powell, 1958). Competition is best studied by considering two different organisms in an environment which has a single growth-limiting nutrient. Three assumptions have to be made:

- (1) That there are no interactions occurring between the two populations which are likely to modify or stabilise the kinetics of free competition.
- (2) The growth environment is constant and ideal in all other respects.

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- (1) That there are no interactions occurring between the two populations which are likely to modify or stabilise the kinetics of free competition.
- (2) The growth environment is constant and ideal in all other respects.

(3) That both organisms respond to the growth conditions or any growth-induced alteration in these conditions in an identical fashion.

When two organisms are competing for the same growth-limiting substrate and no other interactions between the organisms occur, their behaviour can be predicted from known relationships between substrate concentration, s , and the growth rate, μ , (Equation 1.5). Since the substrate limitation restricts an organisms' growth rate (Monod, 1942) the population able to sustain the highest growth rate has the competitive advantage. Further, in open culture systems it is inevitable that those populations which are less competitive are eliminated from the growth environment. In this case the second growth parameter, the saturation constant, K_s , also becomes an important factor in determining the outcome of competitive growth since the organism with the greater affinity for a substrate is at a competitive advantage at lower substrate concentrations (Jannasch, 1968).

Consider an organism A growing in a chemostat culture at a fixed dilution rate, D , and in a steady state at which the growth-limiting substrate is \bar{s} . From equation 1.12

$$D = \frac{\mu_{mA} \bar{s}}{(K_{sA} + \bar{s})} \quad (1.16)$$

In the event of a second organism B being introduced in to the growth vessel, initially as a minor population, then, since the substrate concentration \bar{s} is constant and determined by A, during the first stages of growth of the two-membered culture, the growth of population B is given by:

$$\frac{dx_B}{dt} = (\mu_B - D)x_B$$

$$\text{and so } \frac{dx_B}{dt} = \left[\frac{\mu_{mB} \cdot \bar{s}}{(K_{sB} + \bar{s})} - D \right] x_B \quad (1.17)$$

There are three basic cases to consider in assessing whether or not the growth of population B is more or less competitive than that of the established population A:

(1) For the new population to succeed in ousting population A, then dx/dt has to be positive, an eventuality achieved if $\mu > D$ and so, by considering equation 1.16 and 1.17:

$$\frac{\mu_{mB} \cdot \bar{s}}{(K_{sB} + \bar{s})} > \frac{\mu_{mA} \cdot \bar{s}}{(K_{sA} + \bar{s})}$$

this pertains if either $\mu_{mB} > \mu_{mA}$ (Figure 1.5.1) or $K_{sB} < K_{sA}$ (Figure 1.5.2.) However it must be noted that it is the combined effect of the two parameters which is important in determining whether or not organism B is more competitive than organism A. It is possible to envisage a situation in which $\mu_{mB} > \mu_{mA}$ but $K_{sB} > K_{sA}$ (Figure 1.5.3.). For this pair of organisms, at any growth-limiting substrate concentrations, organism B is the more competitive organism sustaining a higher growth rate than organism A. Initially the rate of growth of organism B is determined by the steady-state concentration established by A; that is at a dilution rate of D the growth-limiting substrate concentration is \bar{s} . Gradually as the proportion of the two

populations begins to change in favour of population B then \bar{s} begins to decrease and tend towards \bar{s}_1 (Figure 1.5.1 and 1.5.2) which is the growth-limiting substrate concentration supporting a growth rate of $\mu_B = D$. At this substrate concentration $\frac{dx_A}{dt}$ must be negative and so population A is unable to grow at the imposed dilution rate and must continue its wash out of the culture vessel.

(2) Population B does not replace A if $\mu_B < D$ and so $\frac{dx_B}{dt}$ is negative, a situation which results if:

$$\frac{\mu_{mB} \bar{s}}{K_{sB} + \bar{s}} < \frac{\mu_{mA} \bar{s}}{(K_{sA} + \bar{s})}$$

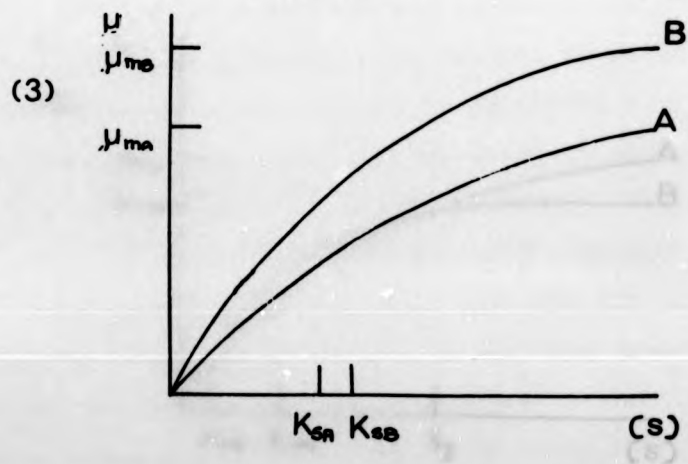
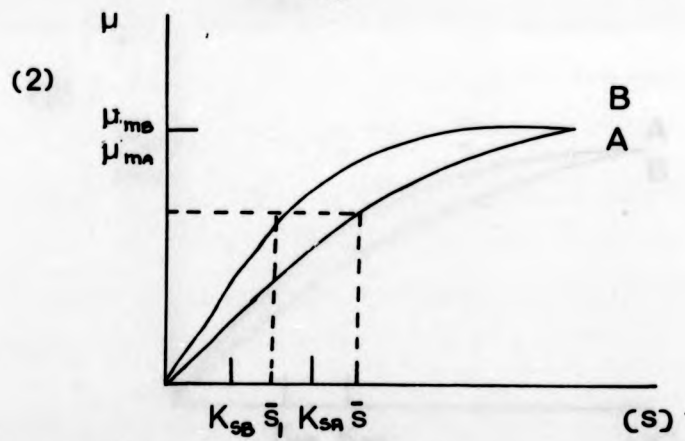
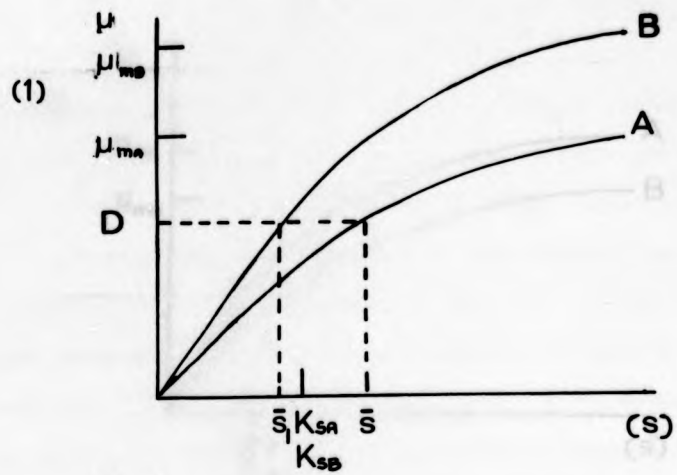
and occurs when either $\mu_{mB} < \mu_{mA}$ (Figure 1.5.4) or $K_{sB} > K_{sA}$ (Figure 1.5.5.). The reverse reasoning to the first case applied for the non-competitive population B.

(3) It is theoretically possible that a stable mixture of two different populations may be set up growing on a single growth-limiting substrate because

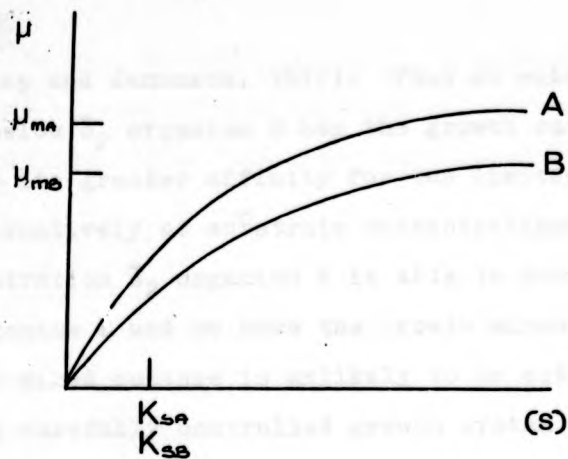
$$\frac{\mu_{mB} \bar{s}}{(K_{sB} + \bar{s})} = \frac{\mu_{mA} \bar{s}}{(K_{sA} + \bar{s})}$$

This is unlikely to occur because it would mean that $\mu_{mA} = \mu_{mB}$ and $K_{sA} = K_{sB}$. What is more likely is that at one particular substrate concentration the growth rates of the two organisms may be identical even though $\mu_{mA} \neq \mu_{mB}$ and $K_{sA} \neq K_{sB}$ (Figure 1.5.6.) For such a special case to occur the two organisms must exhibit Monod crossover

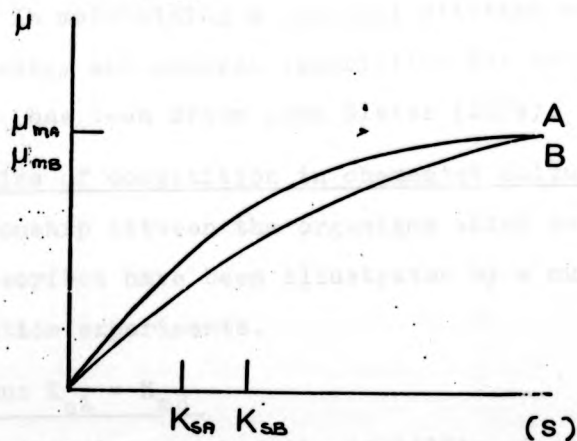
Figure 1.5. Schematic representation of the effect of growth rate μ on substrate concentration $[S]$ for organisms A and B (redrawn from Slater, 1979)



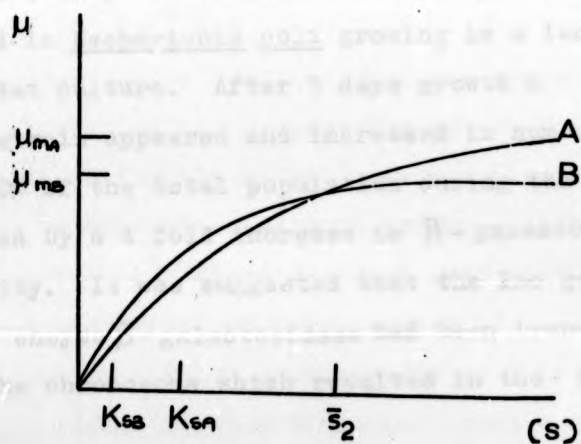
(4)



(5)



(6)



Kinetics (Veldkamp and Jannasch, 1972). Thus at substrate concentrations below \bar{s}_2 organism B has the growth rate advantage due to its greater affinity for the limiting substrate. Alternatively at substrate concentrations above substrate concentration \bar{s}_2 organism A is able to grow more rapidly than organism B and so have the growth advantage. Even so a stable mixed culture is unlikely to be established even in the most carefully controlled growth system because of difficulties in maintaining a constant dilution rate.

The terminology and general description for sections 1.7.1 to 1.7.7 has been drawn from Slater (1979).

1.7.7.1. Examples of competition in chemostat culture.

The relationship between the organisms which have already been described have been illustrated by a number of mixed population experiments.

$$\underline{\mu_{\max A} > \mu_{\max B} \quad \text{but} \quad K_{SA} = K_{SB}}$$

Slater and Bull (1978) described the stability of an F lac plasmid carried in Escherichia coli growing in a lactose-limited chemostat culture. After 5 days growth a plasmid-minus strain appeared and increased in number to form over 90% of the total population during the next 72 h accompanied by a 4 fold increase in β -galactosidase specific activity. It was suggested that the lac gene coding for the enzyme β -galactosidase had been transferred to a site on the chromosome which resulted in the increased

enzyme activity and this produced a 14.5% increase in growth rate of the evolved organism and accounted for its competitive advantage over the plasmid-carrying parent.

$$\mu_{\max A} > \mu_{\max} \text{ and } K_{SA} > K_{SB}$$

Jannasch (1968) was amongst the first to demonstrate that the outcome of competition depended to some extent on the growth rate of the enrichment culture. He studied the behaviour of a Spirillum sp. and E. coli in lactose-limited sea water culture. Above $D = 0.29 \text{ h}^{-1}$ and a lactate concentration 5.0 mg l^{-1} the coliform grew faster and was the successful population. At lower growth rates the Spirillum species grew more efficiently and became the successful organism due to the possession of the high substrate affinity (low K_s value) mechanism for the substrate, thereby exhibiting crossover Monod kinetics (c.f. Figure 1.5.6.).

$$\mu_{\max A} > \mu_{\max B} \text{ but } K_{SA} < K_{SB}$$

Mason and Slater (1979) described how one member of a mixed population can by-pass the growth-limiting substrate restricting the growth rates of the two populations. A tyrosine auxotroph grown in tyrosine-limited chemostat at a fixed dilution rate produced a few revertant prototroph organisms after 84 h growth. This prototroph population grew rapidly at a rate close to μ_{\max} since the specific growth rate was not dictated by the tyrosine concentration.

1.7.8. Calculation of the specific growth rate of the uncompetitive population.

The specific growth rate μ_1 of the uncompetitive population A, during washout can be calculated in two ways.

Firstly using a modified washout growth equation due to Jannasch (1969)

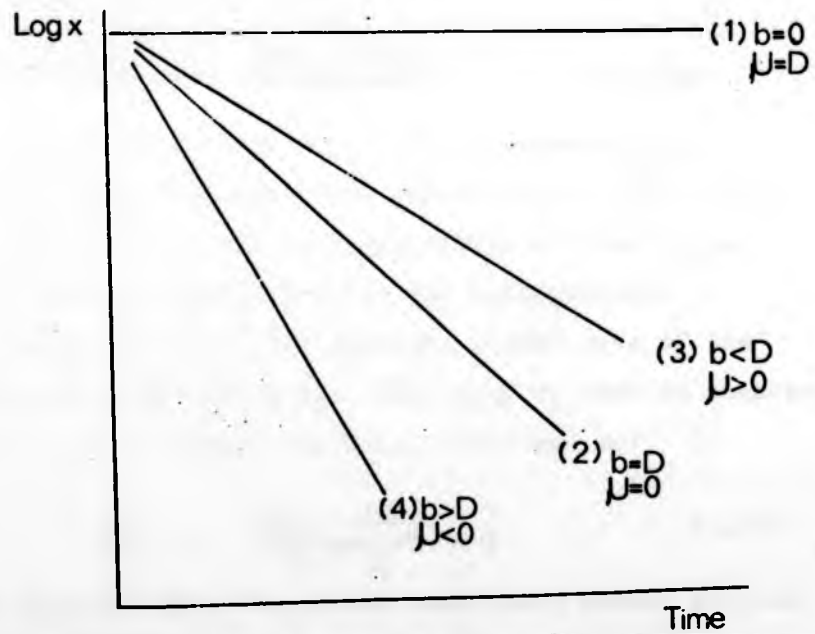
$$\mu_1 = \frac{\ln x_t - \ln x_0}{t_2 - t_1} + D \quad (1.18)$$

where x_0 is the initial uncompetitive population size at time, t ; x_t is the final uncompetitive population size at time t_2 and, D , is the dilution rate maintained for the duration of the experiment. The term $\frac{\ln x_t - \ln x_0}{t_2 - t_1}$

represents a straight line, if the growth conditions remain constant, and the slope, b , equals the rate of washout for the organism A and therefore the difference in growth rates of two populations. Thus there are four possible conditions to be considered:

- (1) If organism A does not washout then $\mu_1 = D$ and $b = 0$ (Figure 1.6).
- (2) If the inoculated population A does not grow then washout continues at a rate equal to the dilution rate such that $b = D$ and $\mu_1 = 0$ (Figure 1.6).
- (3) Organism A may washout at a rate lower than D such that $b < D$ and $\mu_1 > 0$ (Figure 1.6).
- (4) The organism A may washout at a rate higher than the dilution rate D such that $b > D$ and $\mu_1 < 0$.

Figure 1.6 A schematic diagram of the fate of
a potentially uncompetitive population from a
two membered mixed culture.



11 not 11

This can occur if the organism is washing out as a non-growing population due to lysis or bacteriocidal effects of the successful population (Figure 1.6).

Secondly the difference in the specific growth rate of the two competing populations can be calculated from the ratio of the uncompetitive to the competitive populations during washout:

$$\mu_1 - \mu_2 = \frac{\ln R_t - \ln R_0}{t_2 - t_1} \quad (1.19)$$

where R_0 is the initial ratio of the uncompetitive population to the competitive population at t_1 ; R_t is the final ratio of the two populations at time t_2 ; μ_1 is the specific growth rate of the uncompetitive population, x_1 and μ_2 the specific growth rate of the competitive population, x_2 . When $x_2 \gg x_1$ then in chemostat culture $\mu_2 = D$ (Slater and Bull, 1978) and so:

$$\mu_1 = \frac{\ln R_t - \ln R_0}{t_2 - t_1} + D \quad (1.20)$$

Again assuming that the growth conditions remain constant for the observed period of growth then the relationship $\ln R$ against time t yields a straight line, the slope of which gives the difference between the specific growth rates of the two populations.

1.8. THE AIMS OF THE STUDY

The in vitro system thus provides two main advantages over the in vivo experimental system. The use of the

continuous-flow culture system enables the effect of the different environments and selection pressures on the survival of plasmids to be investigated in a more controlled environment than can be provided by the in vivo situation, whilst still achieving a situation close to that found in natural environments. Also the direct effect of the plasmid on the host organism involved in competition with isogenic plasmid-minus strains can also be studied.

Therefore the aims of this investigation can be summarised as :

- (1) To examine the stability of a drug resistant plasmid within populations of E. coli K12 growing in chemostat culture in nutrient-limited conditions and in the absence of antibiotic selection pressure.
- (2) To study changes produced in the plasmid during growth in continuous-flow cultures in carbon and phosphorus limited conditions.
- (3) To examine the ability of plasmid-carrying organisms to survive in direct competition with an isogenic plasmid-minus strain and evolved strains with fewer drug resistance markers in nutrient-limited chemostat culture.
- (4) To investigate the effect of the plasmid on the host organism's physiology and metabolism.

2. MATERIALS AND METHODS

2.1. ORGANISMS AND PLASMIDS.

The parent organism used in this study was Escherichia coli K12 F⁻ strain 1R713 containing the autotransferable plasmid TP120 (R46 or R Brighton) (Grindley et al., 1973). In this study the strain was designated E. coli K12(TP120), and was kindly supplied by Dr. G. Willshaw (Public Health Laboratory, Colindale, London). The plasmid TP120 of compatibility group N coded for resistance to ampicillin (Ap), streptomycin (Sm), sulphonamide (Su) and tetracycline (Tc).

During continuous-flow culture selection experiments (Section 2.9) the following resistant strains were isolated; (1) from a carbon-limited chemostat at a dilution rate $D = 0.1 \text{ h}^{-1}$, E. coli K12(TP120A) (Ap, Sm, Su). (2) from phosphate-limited chemostats all at the dilution rate $D = 0.1 \text{ h}^{-1}$, E. coli K12(TP120B) (Sm, Su, Tc), E. coli K12(TP120C) (Ap, Sm, Su), E. coli K12(TP120D) (Sm, Su) and a plasmid-minus strain E. coli K12(TP120E) (noplasmid), (Section 3.2).

The nalidixic acid resistant strain Escherichia coli K12 711 Nal^R (J62 Nal-r, Harden and Meynell (1972)) used ^{for} as a recipient in transfer experiments (Section 2.10.2) was kindly provided by Dr. K. P. Flint (University of Warwick, Coventry).

Other plasmids used as DNA molecular weight markers in plasmid measurement by agarose electrophoresis (Section 2.13.2) are summarised in Table 2.1. Escherichia coli K12 strain W3110 carrying the plasmid ColE1-K30 was kindly

supplied by Dr. K. G. Hardy, (University of Kent, Canterbury) and all other strains by Dr. K. P. Flint, (University of Warwick, Coventry) or Dr. J. W. Dale, (University of Surrey, Guildford) as indicated in table 2.1.

2.2. CULTURE MAINTENANCE

The strains of Escherichia coli K 12 were maintained on nutrient agar slopes and plates containing appropriate drugs at the following concentration, ($\mu\text{g ml}^{-1}$); ampicillin, 10; streptomycin, 10; sulphonamide, 10; tetracycline, 10; kanamycin, 10; chloramphenicol, 10; Nalidixic acid was added to the final concentration of $50 \mu\text{g ml}^{-1}$ from 1% (w/v) stock solution in 0.1 M NaOH. Oxoid-nutrient agar was used at the recommended concentration of 20 g l^{-1} , autoclaved $15 \text{ lbf in } -2$ for 20 min. and dispensed as agar plates or into 10ml Pyrex glass test tubes fitted with polypropylene caps. Agar plates and slopes were incubated for 24 h at 37°C and stored at 4°C . Agar plates were subcultured every 14 d and agar slopes every 28 d.

2.3. GROWTH MEDIA

For closed culture growth the organisms were grown in the following media:

2.3.1 Nutrient broth.

Oxoid nutrient broth was used at the recommended concentration 14 g l^{-1} and autoclaved $15 \text{ lbf in } -2$ for 15 min.

Table 2.1. List of plasmid-carrying *Escherichia coli* K12 used as DNA molecular weight markers.
 a = provided by Dr. K. P. Flint: b = provided by Dr. J.W.Dale.

	Strain No.	Plasmid	Markers	Size x 10 ⁶ daltons.	References
<u>E. coli</u> K12	W 3110	Col EI-K30	Col ⁺	4.8	Hardy (1975)
<u>E. coli</u> K12	J5-3	a R6K	Tra ⁺ , Ap, Sm.	26	Heffron (1975). Matthew and Hedges (1976).
<u>E. coli</u> K12	J5-3	R1	Tra ⁺ , F1 ⁻ , Ap, Cm, Km, Sm, Su.	62	Clowes, (1972).
<u>E. coli</u> K12	J5 2345	R1 - 19	Tra ⁺ , Fc ⁻ , Dps, Ap, Cm, Km, Sm, Su.	62	Meynell and Datta, (1967).
<u>E. coli</u> K12	J5-3	b pKM101	Tra ⁺ , F1 ⁻ , Ap.	23.7	Mortelmans and Stocker, (1978)
<u>E. coli</u> K12	J5-3	b pACYC 184	Cm, Tc.	2.65	Chang and Cohen, (1977).

2.3.2. Davis and Mingioli medium

Davis and Mingioli medium (1950) contained in glass distilled water, gl^{-1} ; KH_2PO_4 , 2; K_2HPO_4 , 7; $(\text{NH}_4)_2\text{SO}_4$, 2; $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, 0.2; MgSO_4 dehydrated, 0.1. The basic mineral salts medium (pH 7.2) was autoclaved at 15 lbf in $^{-2}$ for 15 min. A 10% (w/v) glucose solution was autoclaved at 10 lbf in $^{-2}$ for 10 min. and after cooling added to cold basic mineral salts medium to give a final concentration of 0.5% (w/v). For carbon-limited continuous-flow culture glucose was added aseptically to a growth limiting concentration of 0.2 gl^{-1} (Section 2.7.1).

2.3.3. Hershey's glucose salts medium

For phosphorus-limited growth the organisms were grown in Hershey's glucose mineral salts medium (Hershey, 1955) which contained in glass distilled water, gl^{-1} : Tris, 12.1; NaCl, 5.4; KCl, 3.0; NH_4Cl , 1.1; CaCl_2 , 0.11; MgCl_2 , 0.095; NaSO_4 , 0.5; FeCl_3 , 0.00016. The salts solution was adjusted to pH 7.4 with HCl prior to autoclaving at 15 lbf in $^{-2}$ for 15 min. Phosphate (KH_2PO_4) was added at the limiting concentration 0.005 gl^{-1} for continuous-flow culture (Section 2.7) or at 0.5 gl^{-1} for closed culture growth (Section 2.4). The stock phosphate solution, 1.0 gl^{-1} , was autoclaved separately at 15 lbf in $^{-2}$ for 15 min. and added after cooling to the cold mineral salts solution. 17% (w/v) glucose was also autoclaved separately at 10 lbf in $^{-2}$ for 10 min. and after cooling added to the mineral salts medium to give a 1% (w/v) final concentration.

2.3.4. Solid media.

Lab M agar (London Analytical and Bacteriological Media Ltd., London) was added to the basic mineral salts medium at the concentration of 1.5% (w/v) and autoclaved at 15lb^f in ⁻² for 20 min. Glucose and phosphate additions were made as in Section 2.3.3.

2.4. GROWTH IN CLOSED CULTURE

The maximum specific growth rate (μ_{\max} , h⁻¹) was determined for the parent and evolved Escherichia coli strains in nutrient broth and in the two defined media (Section 2.3) both in the presence and absence of drugs. 5ml overnight culture was inoculated into 75 ml of medium contained in a 250ml glass conical flask to give an initial culture absorbance (520nm) of approximately 0.01. The cultures were incubated at 37°C with vigorous aeration on a Gallenkamp orbital shaker and growth determined by absorbance measurements at 520nm in a Corning Model 252 colorimeter using sterile media as a blank. μ_{\max} values were calculated from the slope of the exponential phase of growth, using the following equation:-

$$\mu_{\max} = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \quad (2.1.)$$

2.5 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF DRUGS

The minimum inhibitory concentration (MIC) of each drug was determined by the serial dilution tube method (Gould & Bowle (1952)). 0.1 ml overnight culture of E. coli K12(TP120) was inoculated into duplicate Pyrex glass test tubes containing 4.9 ml nutrient broth, Davis and Mingioli medium or Hershey's glucose salts

medium with drugs added separately at concentrations ranging from 0 to $1000 \mu\text{g ml}^{-1}$, control tubes contained either medium without drugs or uninoculated medium. The tubes were incubated for 24 h at 37°C and the absorbance at 520nm determined in a Corning Model 252 colorimeter using sterile medium as a blank.

The minimum inhibitory concentrations of the drugs were the lowest concentrations of the drugs which prevented growth of bacteria. The values for E. coli K12(TP120) were as follows; $\mu\text{g ml}^{-1}$: ampicillin, 80; streptomycin, 15; sulphonamide, 15; and tetracycline, 40.

2.C. DETERMINATION OF FREQUENCY OF PLASMID MARKER LOSS IN CLOSED CULTURE.

An overnight culture of E. coli K12(TP120) grown separately in nutrient broth and the two defined media (Section 2.3) was serially diluted in $0.1\text{M KH}_2\text{PO}_4/\text{KOH}$ buffer pH 7.0 at 37°C to give approximately 100 colonies per agar plate. The 100 colonies were replica plated onto 20 nutrient agar, Davis and Mingioli agar or Hershey's glucose salts agar plates (Section 2.3.4) containing separately the drugs at 60% of the minimum inhibitory concentration, μgml^{-1} : ampicillin, 50; streptomycin, 10; sulphonamide, 10; and tetracycline, 25. Colony counts were determined after 24 h at 37°C for nutrient agar plates and 2 d for defined media agar plates. The frequency of marker loss was expressed as number of sensitive colonies in a population of 2×10^3 resistant cells.

2.7. CONTINUOUS-FLOW CULTURE.

Figure 2.1. illustrates the basic features of the continuous-flow system used in this study to provide the controlled phosphorus and carbon-limited growth of E. coli K12.

The growth vessel (gv) was a Pyrex glass vessel with a working culture volume of 1l. The culture vessel lid which had 5 inlet and outlet ports was fitted to the vessel by a greased, groundglass joint and clamped in position by a metal spring (s). The culture vessel lid ports were fitted with silicon rubber stoppers carrying 2mm or 5mm diameter stainless steel tubing connected to silicon rubber tubing for the supply of gas and medium and the removal of samples and waste. The fifth port was fitted with a Quickfit cooling finger (cf). The culture vessel contained a 5cm magnetic stirrer bar (sb) and was positioned on a Heindolph magnetic stirrer so that a homogeneous suspension was maintained at 1000rpm. The culture was continuously sparged by air at a rate of 1l min⁻¹ through a scintered glass sparge. The air was passed through an in-line standard sterile glass filter made from expanded glass tubing filled with glass wool and plugged with cotton wool (f.4). Temperature of the culture was maintained at 37°C by the cooling finger connected to a Churchill pump (Instrument Co., Middlesex, England.). Fresh medium was supplied from a 20l Pyrex glass vessel (mv), fitted with a silicon rubber stopper which carried two stainless steel tubes and was held securely in place by two aluminium plates bolted together. One tube was connected to a single sterile glass

filter (f 1) to allow flow of sterile air into the vessel as medium was transferred to the culture vessel. Sterile fresh medium was pumped into the growth vessel by a peristaltic flow inducer (pp), (MHRE7, Watson - Marlow Ltd, Falmouth, Cornwall, England.). Fresh media vessels could be connected at the metal connecting hood (h). Flow of medium into the culture vessel was measured by passing the medium supply through a 25ml pipette (p) attached to the main medium line by a Portex T piece and which was clipped off during normal medium flow. Waste medium and dead organisms were removed under pressure into a 20l Pyrex glass vessel which was disconnected and replaced with an empty sterile vessel when full.

2.7.1 Culture samples

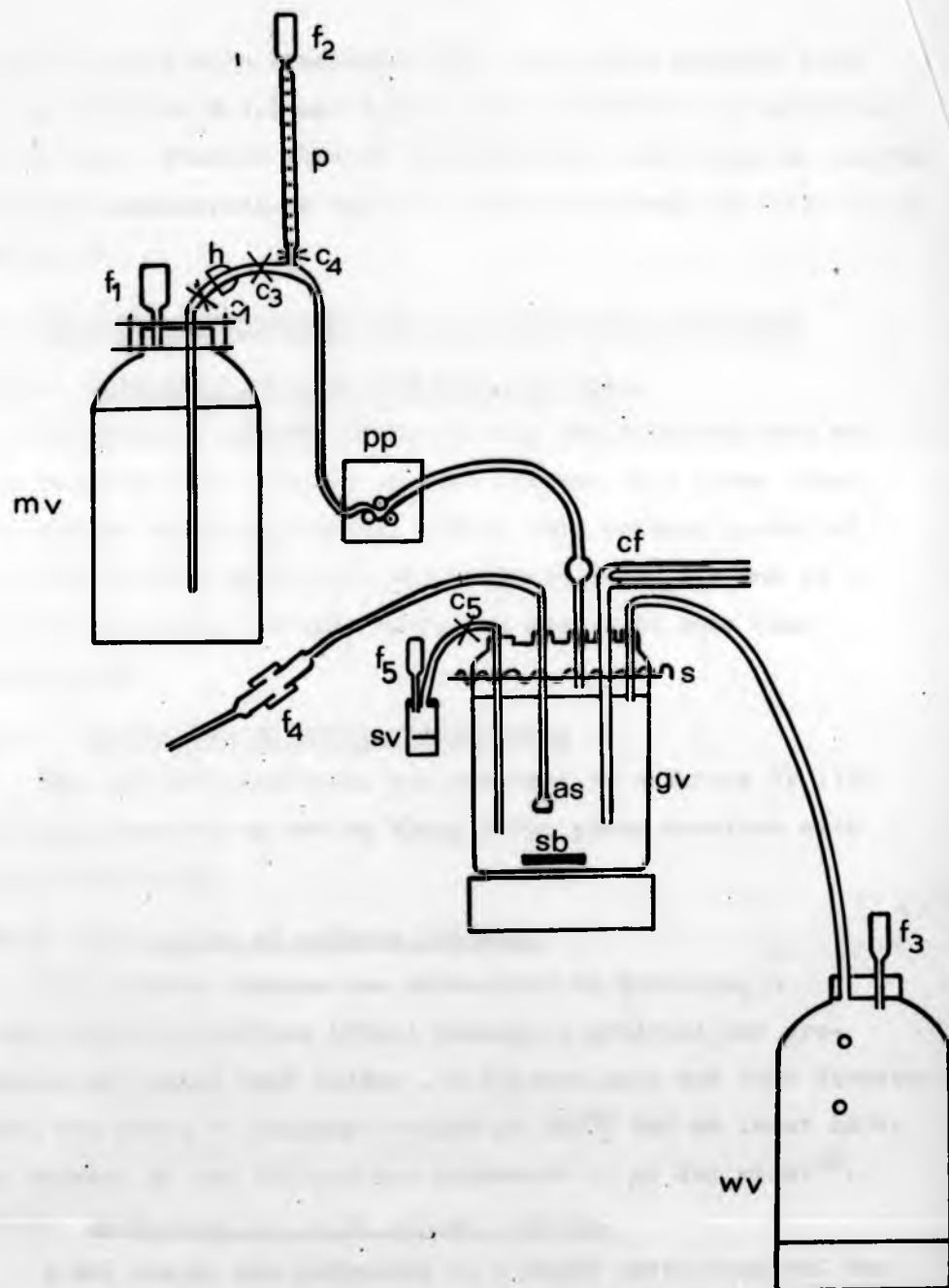
Samples were removed into a 1oz McCartney glass bottle (sv) fitted with a sterile glass filter. This was achieved by opening the Hoffman clip (c₅) while the outlet tube to the waste pot was closed by clip (c₆). The culture sample then flowed into the McCartney bottle under pressure.

2.7.2. Sterilisation of continuous-flow culture system.

The empty waste vessel was connected to the culture vessel for sterilisation. The media vessel was disconnected at the connecting hood (h) which was covered with aluminium foil. All Hoffman clips, except that on the waste tubing, were closed and all air filters covered with aluminium foil. The system was autoclaved at 15lbf in⁻² for 15 min.

The media vessel was autoclaved separately with the Hoffman clips, c₁, closed and the silicon tubing and air

Figure 2.1. Schematic diagram of the continuous-flow culture system showing: growth vessel (gv); metal spring (s); stirrer bar (sb); air sparge (as); sample vessel (sv); cold finger (cf); media vessel (mv); pipette (p); peristaltic pump (pp); waste vessel (wv); Hoffman clips (c_1, c_2, c_3, c_4, c_5); air filters (f_1, f_2, f_3, f_4).



filter covered with aluminium foil. The basic mineral salts medium (Section 2.3.2 and 2.3.3) was autoclaved for 151bf in⁻² for 40 min. Sterile glucose and phosphate were added at growth-limiting concentrations when the medium was cold (Section 2.3.2. and 2.3.3).

2.8. ANALYTICAL PROCEDURES FOR CONTINUOUS-FLOW CULTURES

2.8.1. Estimation of a steady-state culture.

To obtain a steady- state culture the dilution rate was kept constant for a period of time greater than three times the culture doubling time, t_d . After this initial period of time the culture absorbance was measured every 2 h and if it remained constant a steady-state was deemed to have been established.

2.8.2. Estimation of culture absorbance

The culture absorbance was measured in a Unicam SP 1700 spectrophotometer at 520 nm using 1.0ml glass cuvettes with a 1cm light path.

2.8.3. Estimation of culture biomass.

The culture biomass was determined by filtering a known volume of culture (20ml) through a predried and pre-weighed millipore HAWP filter , 0.45µ pore size and 22mm diameter which was dried to constant weight at 105°C for at least 18 h. The biomass of the culture was expressed in µg dry wt ml⁻¹.

2.8.4. Estimation of total organic carbon.

A 4ml sample was harvested in a bench centrifuge and the pellet resuspended and washed in sterile distilled water. The washing was repeated and the pellet resuspended and serially

diluted in sterile glass distilled water to give a concentration between 20 to 80ppm total organic carbon which was determined in a Beckman Total Organic Carbon Analyser, model number 915-B, (Beckman, U.S.A.)

2.8.5. Estimation of viable cell number and drug sensitivity.

Routinely samples were diluted appropriately in 0.1 M $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer pH 7.0 at 37°C and 0.1ml samples plated out on nutrient agar plates containing drugs separately at $\mu\text{g ml}^{-1}$ ampicillin, 50; streptomycin, 10; sulphonamide, 10; or tetracycline, 25. Viable counts were determined after 24 h for nutrient agar plates and 48 h for defined medium plates. Results were expressed as viable organisms ml^{-1} .

2.8.6. Estimation of total cell number.

For viability studies (Section 5.) total cell number was estimated by means of an electron particle counter (Model 2131 Coulter counter) with optical instrument settings as follows:- window width, 100; aperture size, 30μ ; aperture current (I), $\frac{1}{2}$; and amplification (A), $\frac{1}{8}$. Culture samples were serially diluted in freshly filtered ISOTON (ISOTON II, without azide, Coulter Electronics, England). Dilutions of 1: 100 to 1: 800 were usually suitable and results expressed as total number of organisms ml^{-1} .

2.8.7. Estimation of cell volume.

Cell volume was estimated by the electronic particle counter for the same samples as for cell number determination, by use of the Coulter Channelyser. In this method cell size was related to a channel number over the range 1 - 100 and the

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Cell volume was estimated by the electronic particle counter for the same samples as for cell number determination, by use of the Coulter Channelyser. In this method cell size was related to a channel number over the range 1 - 100 and the

distribution display of the Coulter Channelyser gave a distribution curve of cell size against cell number.

The channel number at the peak of the distribution was determined and the mean cell volume, V , calculated from the formula

$$V = (\text{channel number}) \times \frac{WW}{100} + BCT \times T_f.$$

where WW = window width set to get maximum cell number count (normally setting of 100)

BCT = base channel threshold, which determined the smaller particle size to be included in the distribution analysis (normally 10 or 15)

T_f = threshold factor determined by the calibration of the Coulter Channelyser using a standard particle volume i.e. 0.807 μm diameter latex beads (Calibration Latex, Coulter Electronics Ltd., Herts, England.)

Results were expressed as volume in cubic microns.

2.9 PROCEDURE FOR SELECTION EXPERIMENTS IN CONTINUOUS-FLOW CULTURE

For the selection experiments, chemostats were inoculated with 10 ml of an overnight culture of E. coli K12(TP120) and grown in batch culture for several hours. For carbon-limited growth Davis and Kingioli medium (Section 2.3.2) was used and for phosphate-limited growth Hershey's glucose salts media (Section 2.3.3) supplemented with drugs at the same concentration as stock slopes, $\mu\text{g ml}^{-1}$; ampicillin, 10; streptomycin, 10; sulphonamide, 10; tetracycline, 10. The fresh medium flow was initiated to give the required dilution

rate and initially a steady state culture was established in the presence of drugs (2.8.1). The medium was changed to one lacking the drugs and samples taken at 24 h intervals for viable organism and drug sensitivity determinations (Section 2.8.5). This procedure determined the number of resistant organisms in the whole bacterial cell population within the chemostat.

2.10. DETERMINATION OF PLASMID MARKERS

2.10.1 Determination of plasmid drug resistance markers.

Drug resistance markers carried by evolved strains isolated from continuous-flow culture selection experiments (Section 2.9) were determined. Organisms which had been isolated as single colonies, on nutrient agar plates in the course of the experiment were picked off and streaked onto nutrient agar plates containing drugs at 60% of the minimal inhibitory concentration (Section 2.3). why?

2.10.2. Determination of the transfer marker.

The maintenance, or otherwise, of an autotransferable plasmid in the various isolates was demonstrated by its potential to transfer to a plasmid-minus E. coli recipient using a modified plating method of Dennison and Baumberg (1975). Cultures of the recipient E.coli K12 711 NaI^R were grown in nutrient broth to the end of the exponential phase giving approximately 5×10^9 organisms ml⁻¹. Donor cells were grown to mid-exponential phase in either nutrient broth or Davis and Mingioli medium or Hershey's glucose salts medium to give approximately 2×10^8 organisms ml⁻¹.

0.1ml of the nalidixic acid resistant recipient was spread on nutrient agar plates supplemented with 10µg ampicillin ml⁻¹ or 10µg streptomycin ml⁻¹ plus 50µg nalidixic acid ml⁻¹. The donor strains were serially diluted in 0.1 M KH₂PO₄ /KOH buffer pH 7.0 at 37°C to 10⁻⁶ dilution and 0.02 ml of each dilution inoculated dropwise onto the surface of plates spread with E. coli Nal^R. To provide control plates, donor and recipient strains were inoculated dropwise onto nutrient agar plates supplemented separately with the antibiotics or nalidixic acid. All plates were incubated for 18 h at 37°C and the number of colonies growing at a suitable dilution were counted. These colonies represented nalidixic acid resistant E. coli organisms which had received by conjugation a plasmid carrying ampicillin or streptomycin resistance (transconjugants). The frequency of transfer was expressed as the percentage of transconjugants to total donor organisms.

For each conjugation experiment, a number of transconjugant colonies were transferred to a range of media supplemented separately with the four drugs, to check for the concomitant transfer of all the expected resistance markers.

2.11. PROCEDURE FOR TWO MEMBERED MIXED CULTURE COMPETITION EXPERIMENTS IN CONTINUOUS FLOW-CULTURE.

Two membered mixed culture experiments were performed with the parent strain E. coli K12(TP120) and five evolved strains (Section 2.1) in separate experiments and also between selected evolved strains.

In a preliminary experiment, an overnight culture of the parent strain E. coli K12(TP120) was inoculated into a carbon-limited chemostat and after a period of closed culture growth, the flow of medium initiated and a steady state culture established. The evolved strain E. coli K12(TP120A) was grown separately overnight in Davis and Mingioli medium and harvested aseptically by centrifugation at 23,000 g for 20 min. and resuspended in 10ml of the carbon-limited medium for inoculation into the chemostat containing the growing, steady state culture of E. coli K12(TP120). This scheme of inoculation was rejected because it was complicated by the harvesting stage and apparently affected the survival of the parent strain causing it to disappear from the population at a faster rate (Section 4.1.).

As an alternative procedure, separate overnight cultures of two strains grown in the appropriate defined medium with the routinely used concentration of drugs (Section 2.3.) were simultaneously inoculated into the chemostat to give approximately equal population densities. After a period of closed culture growth to overcome problems associated with variable lag phases, to ensure that both populations were growing exponentially, the fresh medium flow was initiated to give selected dilution rates ranging from 0.1 h^{-1} to 0.35 h^{-1} . Samples were taken at 3 h and 4 h intervals for viable organism determination (Section 2.8.5) by plating on nutrient agar and nutrient agar supplemented with the appropriate selective drugs. In the case of the parent strain E. coli K12(TP120) (Ap, Sm, Su, Tc,) and the evolved strain E. coli K12(TP120A) (Ap, Sm, Su,) the

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strains were distinguished on tetracycline supplemented plates. An increase or decrease in tetracycline resistant organisms reflected the increase or decrease in the parent strain.

The specific growth rate of the uncompetitive population, μ_1 , was calculated by two methods. Firstly, using a modified washout growth rate equation due to Jannasch (1969)

$$\mu_1 = \frac{\ln x_t - \ln x_0 + D}{(t_2 - t_1)} \quad (1.18)$$

and by an equation due to Slater and Bull (1978)

$$\mu_1 = \frac{\ln R_t - \ln R_0 + D}{(t_2 - t_1)} \quad (1.19)$$

(Section 1.7.8.)

2.12. ISOLATION OF PLASMID DNA

2.12.1. Isolation of plasmid DNA by dye buoyant caesium chloride density gradient centrifugation.

Plasmid DNA for contour length measurements (Section 2.13.1) was isolated by a dye buoyant caesium chloride gradient centrifugation method (Palchaudhuri and Chakrabarty, 1976) as modified by K. G. Hardy, (University of Kent, personal communication).

The plasmid-containing strain was inoculated from stock slopes into 500ml Davis and Mingioli medium containing the appropriate range and concentration of drugs (Section 2.3.) and grown with vigorous aeration at 37°C to approximately 5×10^8 organisms ml⁻¹.

The organisms were harvested by centrifugation at 23,000g

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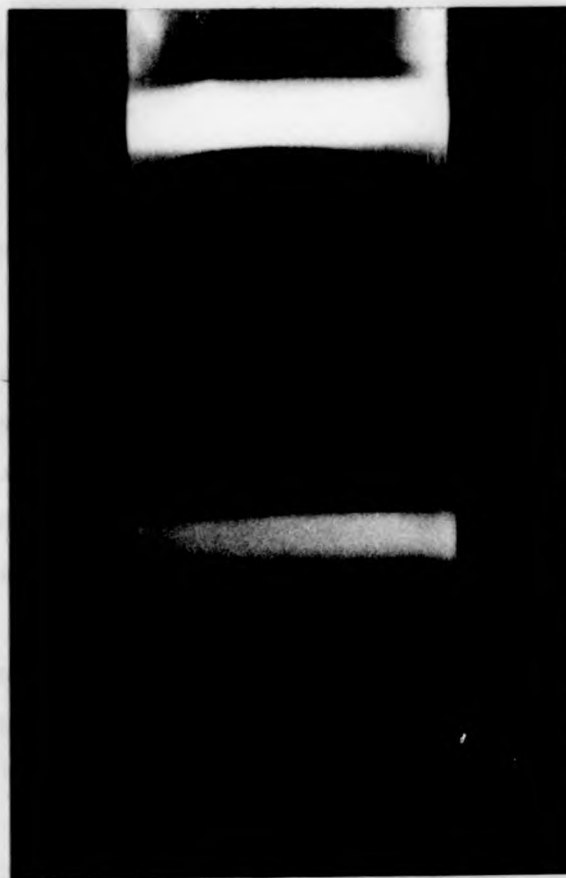
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The organisms were harvested by centrifugation at 23,000g

for 20 min. and resuspended in 25 ml sterile 25% (w/v) sucrose solution in 50 mM Tris-HCl buffer, pH 8.0. 2.5 ml freshly prepared lysozyme (Sigma Chemical Co., London) from a solution of 5 mg ml⁻¹ in 0.25 M Tris-HCl buffer pH 8.0 was added and followed with 2.5 ml 0.25 M ethylenediaminetetraacetate (EDTA). The solution was mixed gently and incubated at 37°C for 30 min. 2.5 ml 5.7% (v/v) Sarkosyl NL35 (Ciba-Geigy, Manchester, England.) in 1.5 M NaCl and 50 mM Tris-HCl buffer pH 8.0 was added slowly to the bottom of the tube with gentle mixing. The lysate was sheared by two passages through a 10 ml disposable syringe. This lysate was centrifuged in an MSE Prep Spin Mark II 65 ultracentrifuge (MSE, Crawley, England) using a 6 x 5.5 ml aluminium swingout rotor at 70,000 g for 10 min. at 20°C. 0.5 ml to 1.0 ml of supernatant was removed from the pellet and termed the cleared lysate. 3.9 ml cleared lysate, 0.12 ml ethidium bromide (1.5% w/v) in 0.5 M Tris-HCl buffer, pH 8.0) and 3.6 g caesium chloride were mixed in a 5.5 ml nitrocellulose tube. The mixture was centrifuged at 170,000 g for 40 h at 15°C using an MSE Prep Spin 65 ultracentrifuge with a 6 x 5.5 ml swing out rotor.

The tubes were examined under short and long waveband UV light and the lower covalently closed circular plasmid DNA band (Figure 2.2) removed with a 1 ml disposable syringe fitted with a gauge 18 needle. The DNA samples were pooled and stored at -20°C. Before contour length measurements for molecular weight determination in the electron microscope (Section 2.13.2) ethidium bromide was removed by shaking with an equal volume of isopropanol equilibrated with caesium

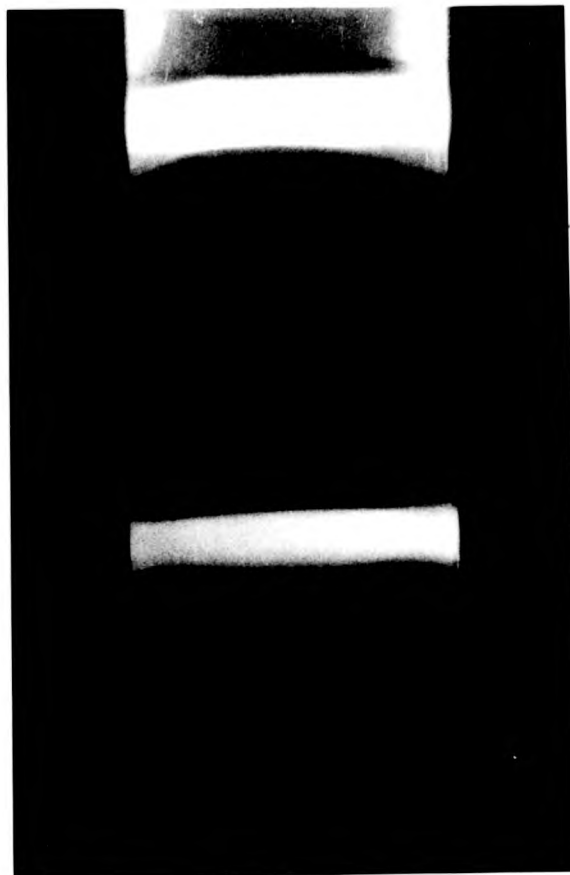
Figure 2.2 Isolation of plasmid DNA on caesium chloride density gradient showing chromosomal DNA and (CCC) plasmid DNA bands.



Meniscus

Chromosomal
DNA

Plasmid DNA



Meniscus

Chromosomal
DNA

Plasmid DNA

sium

el

chloride to approximately the same density as that in the gradient. The dye passed into the alcohol phase but several extractions were necessary to remove all the ethidium bromide. Finally caesium chloride was removed by dialysis against 21 of buffer containing 0.01 M Tris and 0.0001 M EDTA pH 8.0.

2.12.2. Preparation of crude lysates for agarose electrophoresis

Preparation of crude lysates for agarose electrophoresis was by a modified method of Meyers et al., (1976). Plasmid-containing strains were grown overnight in 60 ml nutrient broth (Section 2.3) and harvested by centrifugation at 23,000 g for 20 min. The organisms were resuspended in 3 ml 25% (w/v) sucrose in 10 mM Tris and 1 mM EDTA pH 8.0. Cleared lysates were prepared by a modified sodium dodecyl sulphate (SDS) - salt precipitation method described by Guerry et al. (1973). During the procedure the preparations were contained in Beckman Rotor type JA20, polypropylene tubes (Beckman, U.S.A.) 0.4 ml lysozyme from a solution 5 mg ml⁻¹ in 0.25 M Tris-HCl, pH 8.0 was added and the preparation placed on ice for 5 min. 0.8 ml 0.25 M EDTA was added and the preparation was placed on ice. 1.2 ml 5% (w/v) SDS solution was added, followed by 1.2 ml 6 M NaCl added very slowly into the bottom of the tube. The preparation was left at 4°C overnight and centrifuged at 17,000 g for 30 min. at 4°C in a Beckman, JB21 centrifuge with a JA20 rotor type (Beckman, U.S.A.). The cleared lysate supernatant was removed and the volume doubled by the addition of distilled water. An equal volume of Tris (50 mM) - saturated phenol was added and the tube inverted gently several times and centrifuged at 12,100 g for 30 min. at 20°C

to obtain a clear aqueous phase sample. Rarely was more than one phenol extraction necessary but, if required the loss of plasmid DNA was minimised by collecting the aqueous-phenol interface after each extraction. A number of interface samples were pooled and extracted again as described above and the aqueous phase added to the first aqueous phase sample. The clear aqueous phase sample was brought to 0.3 M sodium acetate (final concentration) and twice the volume 95% (v/v) ethanol at -20°C added to precipitate the plasmid DNA. The tube was stored at -20°C overnight and the precipitated DNA recovered by centrifugation at 12,000 g for 10 min. at room temperature in a micro-angle bench centrifuge (Baird and Tatlock, London). The ethanol was thoroughly drained from the tube and the precipitated DNA was resuspended in 100 μl TES buffer (50 mM NaCl, 5 mM EDTA and 50 mM Tris, pH 8.0). The DNA sample was analysed immediately by agarose gel electrophoresis (Section 2.13.2) or stored at -20°C .

2.12.3. Isolation of pure plasmid DNA for endonuclease digestion

Crude plasmid DNA for endonuclease digestion was prepared by the modified method of Meyers et al. (1976) but if a more purified DNA was required, by the modified method of Thomson et al. (1974).

Organisms were grown overnight in 2 l of nutrient

broth and harvested by centrifugation at 23,000 g and resuspended in 48 ml cold 25% (w/v) sucrose in 0.05 M Tris-HCl, pH 8.0. Lysosyme (6.9 ml of a freshly prepared solution at 10 mg ml⁻¹ in 0.25 M Tris -HCl, pH 8.0) was added and the mixture shaken for 30 min. at 37°C and placed on ice. After 5 min. 12 ml 0.25 M EDTA pH 8.0 was added and after a further 5 min. the organisms were lysed by adding 54 ml 2% (v/v) Triton X - 100 in 0.05 M Tris - HCl pH 8.0, and 0.0625 M EDTA. A period of 20 min. was allowed for lysis and the crude lysate cleared by centrifuging at 17,000 g for 15 min at 4°C in a Beckman JB21 in a JA20 rotor. The supernatant was removed and divided into equal volumes and layered over 3 ml saturated (61.7% (w/v)) caesium chloride in three MSE SW25 centrifuge tubes. The tubes were completely filled by layering distilled water over the cleared lysate. DNA in the cleared lysate was pelleted into the CsCl cushion by centrifugation at 70,000 g for 20 h at 15°C in the MSE Prep Spin 65 ultracentrifuge. The bottom 8 ml of each tube was collected by pipetting and the fractions pooled. Ethidium bromide (10 ~~gl~~ ml⁻¹) was added to give a final concentration of 500 µg ml⁻¹. The density was adjusted to 1.55 g caesium chloride ml⁻¹ (48.4% (w/v) CsCl) in a total volume of 30 ml with CsCl and TES buffer (0.05 M Tris - HCl, 0.05 M NaI and 5 mM EDTA pH 8.0). The DNA was banded at 170,000 g for 24 h at 15°C in an MSE 6 x 5.5 ml

swing out rotor in the MSE Prep Spin 65 ultracentrifuge. The tubes were examined under long and short wave UV light and the plasmid DNA removed by a 1 ml disposable syringe fitted with a gauge 18 needle. These plasmid DNA bands were pooled and recentrifuged in two 5 ml volumes for 20 h. The plasmid bands were collected and ethidium bromide removed by dialysis against 10 g Dowex Na^+ resin in 54 ml buffer (0.8 M NaCl, 50 mM Tris -HCl and 10 mM EDTA pH 8.0). The resin was prepared by washing in 2 M HCl followed by 1 M NaOH to convert it to the Na^+ form. The DNA solution was finally dialysed into TE buffer (10 mM Tris - HCl and 1 mM EDTA pH 7.2). The DNA concentration was calculated spectrophotometrically assuming that an absorbance of 260 nm of 1.0 corresponds to 50 μg DNA ml^{-1} .

2.13. PLASMID DNA MOLECULAR WEIGHT DETERMINATION

2.13.1 Electron microscope contour length measurement of plasmid DNA

The contour length measurements of plasmid DNA were determined using the aqueous technique of Davis et al. (1971). This method used 3.5 mm copper grids (Agar Aids, Caversham, Berks, England.) which has been covered with a freshly spread parlodion film. 3.5% (w/v) Parlodion was baked at 90°C for 24 h before dissolving in n - pentyl acetate by stirring for 24 h.

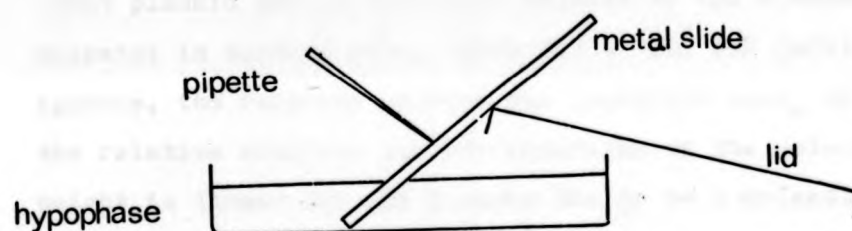
The coating procedure involved the use of a Buchner funnel which had rubber tubing attached to the neck and closed off by a Hoffman clip. The Buchner funnel was rinsed out several times with absolute alcohol and filled with distilled water to a depth of 4 cm. A grid platform was placed in the funnel and copper grids positioned on this platform, with their shiny sides uppermost. A single drop of Parlodion solution was delivered onto the surface of the water and spread out into a very fine thin surface film. The Hoffman clip was opened carefully and water very slowly run out allowing the parlodion film to settle uniformly over the copper grids. A pair of forceps was run around the edge of the grid platform to allow the platform to be lifted free of the film. The grids were ready for use 3 h after coating and remained stable for 48 h.

20 μ l dialysed plasmid DNA was added to a spreading solution which contained : 20 μ l ColE1 standard plasmid DNA for sizing purposes; 10 μ l 6 M ammonium acetate; 1 μ l 50 mM EDTA pH 8.5; 1 μ l 0.5 M Tris pH 8.5; and 8 μ l 2 mg Cytochrome C ml^{-1} in 10^{-5} M EDTA was added just before spreading. The spreading procedure was as follows: A microscope slide shaped strip of stainless steel was heated in a hot flame for approximately 10 min. to produce a very clean surface. The slide was placed at an angle against the upturned lid of a Petri dish and running down into the

Petri dish bottom (Figure 2.3) Approximately 30 ml freshly prepared 0.25 M ammonium acetate, pH 7.5, the hypophase, was poured down the metal slide and allowed to drain before the spreading solution was applied. 50 μ l of the spreading solution was spread evenly across the slide using a glass pipette, starting 1 cm above the surface of the hypophase contained in the Petri dish bottom and ending as the spread solution touched the hypophase. The DNA-protein film was picked up at once onto a prepared grid at 1 grid's distance away from the ramp. The grid was stained for 30 sec. in 5×10^{-5} M uranyl acetate prepared from a stock solution of 5×10^{-2} M uranyl acetate in 10 mM HCl which was diluted 1×10^{-3} in ethanol before use. The grid was rinsed for 2 sec. in 90% (v/v) ethanol followed by a 10 sec. immersion in isopentane.

To improve contrast the grid was shadowed at an angle of 7° with platinum-palladium wire in a high vacuum coating unit with a rotary table (Nantech (Thin Films) Ltd., Manchester, England.) The grids were examined using an AEI Corinth 27 Electron microscope at 10,000 x and 20,000 x magnification with an accelerating voltage of 60 KV. Electron micrographs were taken of fields of view which contained the plasmid TP120 and standard ColE1 - KH30 plasmid DNA using 70 mm line film, N4E50, (Kodak, Liverpool, England.). Negatives were enlarged 10 to 50 times using a film projector and the outline of plasmid DNA traced onto plain paper. Contour lengths were measured using a Jakar map measure model number

Figure 2.3. Schematic diagram of spreading arrangement used to prepare grids for the electron microscope.



120 KK. At this magnification map measuring errors were not significant. A total of 35 molecules were measured by this method and the molecular weight expressed $\times 10^6$ daltons.

2.13.2. Measurement of plasmid DNA molecular weight by agarose gel electrophoresis.

Molecular weight of covalently closed circular (CCC) plasmid DNA is inversely related to the distance migrated in agarose gels. With 0.7 - 0.85% (w/v) agarose, the relation between the logarithm base_e of the relative mobility and the logarithm of the molecular weight is linear for CCC plasmid DNA up to a molecular weight of approximately 90×10^6 daltons.

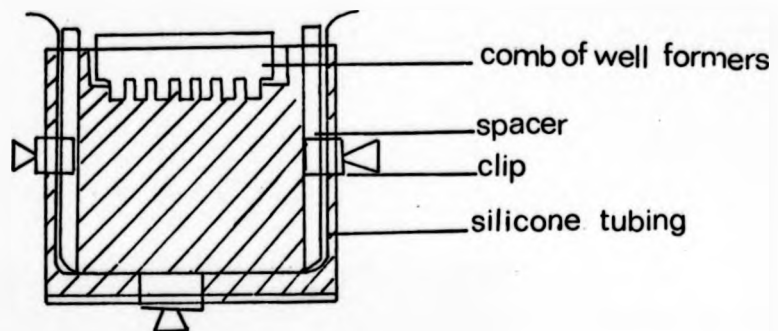
Electrophoresis was performed in vertical slab gels (Raven Scientific Ltd., Suffolk, England) (Figure 2.4.). The glass plates were thoroughly washed with detergent and rinsed several times in distilled water. The spacers were placed 1 cm in from the edge of the notched plate and the 2 mm silicone tubing arranged around them. A second square plate was placed over this and they were clipped together with three 5 cm bulldog clips. 0.7% or 0.8% (w/v) electrophoresis grade agarose (B.D.H. Chemicals, Dorset, England.) was dissolved in 100 ml electrophoresis buffer containing 40 mM Tris, 2 mM EDTA and 20 mM sodium acetate pH 7.8 in a boiling water bath. Agarose was cooled to 65°C and poured into the assembled gel framework and a comb bearing 10 x 5 mm well formers was slotted into the gel and left to set. Sharper bands were

obtained by placing the gel at 4°C for 2 - 3 h before use. When the gel was cold and solid, the silicone tubing and well formers were removed very carefully. The gel framework was secured to the buffer chambers by the same large clips (Figure 2.4). 10 - 50 µl ethanol precipitated DNA samples were adjusted to a concentration of 10% (w/v) sucrose and 0.04% (w/v) bromophenol blue before loading into the gels. The gels were run at a constant voltage of 120 V (50 mA) for 3 - 5 h at 4°C. After electrophoresis the gels were soaked for 30 min. in 0.5 µg ethidium bromide ml⁻¹ and examined under long and short wave ultraviolet light. Chromosomal and plasmid DNA appear as brightly fluorescent red bands on a black background (Figure 2.5).

To photograph the gels they were placed in shallow black Teflon-coated non-stick cake tins which were found to be excellent containers for staining and phototraphy. The gels were illuminated by two angled ultraviolet lamps and photographed on 35 mm Kodak HP5 film using a Praktika MLT3 camera (Pentacore, East Germany) with Hoya 49 mm orange filter and U.V filters. Migration distances (expressed in mm) were measured from the bottom of the well to midpoint of the plasmid DNA band (Figure 2.5). The logarithm of migration distance (relative mobility) of standard plasmid DNA was plotted against the logarithm of molecular weight (Figure 2.6). The molecular weight of plasmid DNA of unknown size was calculated from the standard

Figure 2.4 Schematic diagram of vertical slab gels.

(a) Gel apparatus assembled prior to receiving agarose gel solution. (b and c) Gel apparatus in position in buffer tanks.



o gels.

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ln

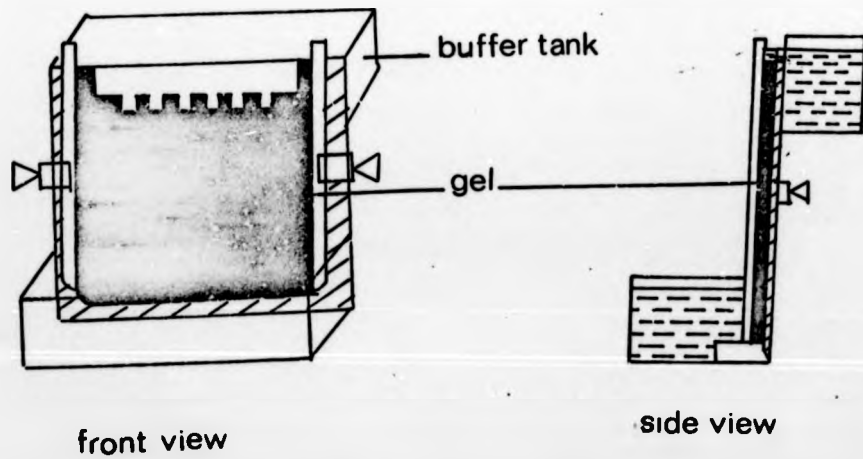
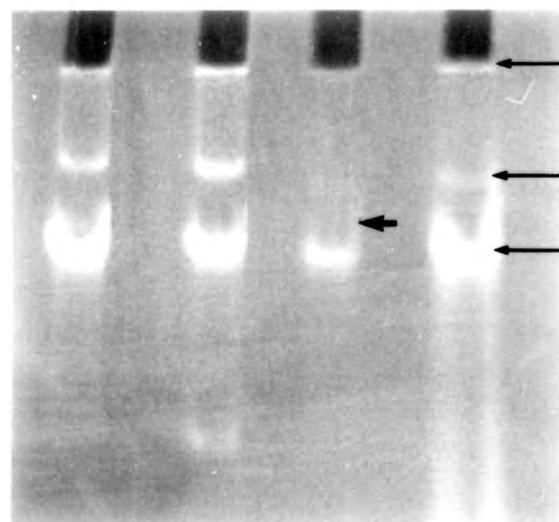


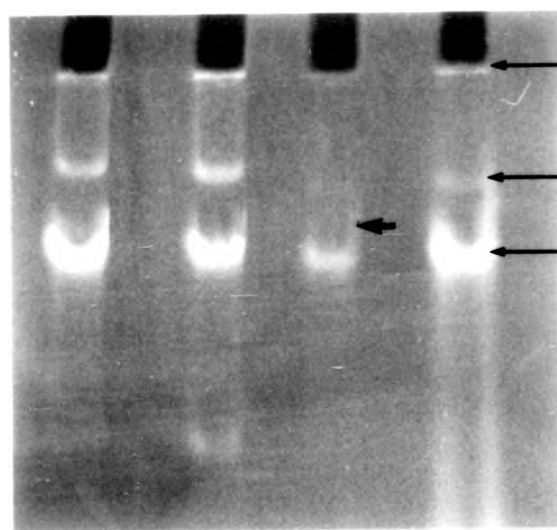
Figure 2.5 Agarose gel electrophoresis of plasmid DNA from E. coli K12 strains showing migration distance. (A) R1 - 19, 30 μ l. (B) Upper band: R 1, 30 μ l; lowest band: ColE1, 20 μ l. (C) R6K, 30 μ l; (D) TP120 , 30 μ l.



Bottom half of gel

Plasmid DNA

Chromosomal DNA

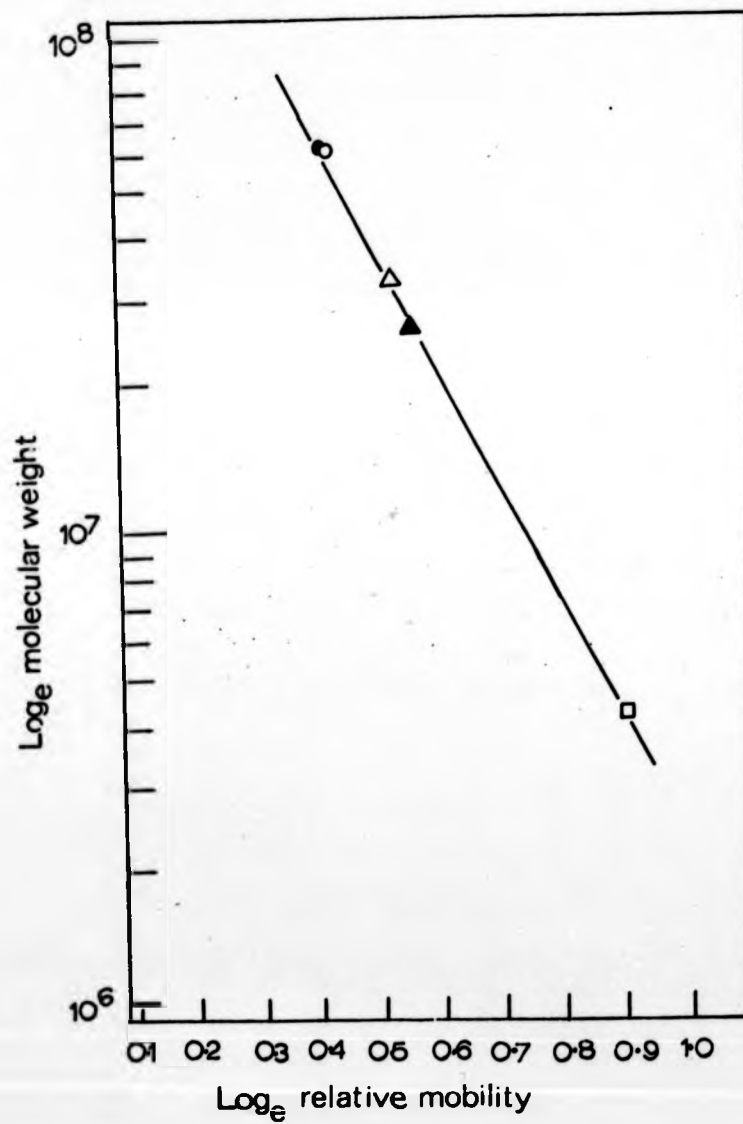


Bottom of gel

Plasmid DNA

Chromosomal
DNA

Figure 2.6. A plot of the \log_e molecular weight against \log_e relative mobility of plasmid DNA from E. coli K12 strains carrying plasmids of known molecular weight. \circ , R1 (MW 62×10^6); \bullet , R1 - 19 (MW 62×10^6); Δ , TP120 (MW 31.7; \blacktriangle , R6K (MW 26×10^6); \square , ColE1 (MW 4.2×10^6).



curve and expressed in daltons.

2.14. ENDONUCLEASE DIGESTION OF PLASMID DNA

Restriction endonucleases are site specific endo-deoxyribonucleases that cleave double stranded and some single stranded DNA. All restriction endonucleases recognise specific DNA sequences and some but not all also cleave at the same sites (Roberts, 1976). All plasmids have a characteristic number of specific sites and on digestion with a particular endonuclease produce a number of different lengths of linear DNA which produce a characteristic pattern of bands following electrophoresis on agarose gels. This has been used to identify resistance plasmids in different bacterial populations (Sadowski, Peterson, Gerding and Cleary, 1978). Plasmids which have undergone evolution or change in DNA which has involved modification of these sites will demonstrate a changed pattern of digestion.

2.14.1. Digestion of plasmid DNA

Plasmid DNA digests were performed using two different endonucleases namely EcoRI (Boehinger, Sussex, England and Miles Laboratories Ltd., Slough, Berks, England) and BamHI (Miles Laboratories Ltd., Berks, England). The digests were carried out according to the manufacturers instructions using 1 ml polypropylene tubes at 37°C for 1 h. A typical incubation mixture contained: 20 µl plasmid DNA solution; 1 µl endonuclease enzyme and 30 µl reaction

buffer containing 0.1 M Tris, 0.1 M NaCl, 10 mM MgCl_2 , 10 mM β mercaptoethanol pH 7.5. EcoRI endonuclease had an activity of 5×10^4 units ml^{-1} which was diluted 1:30 in the dilution buffer. The dilution buffer contained 50 mM KH_2PO_4 , 0.2 M NaCl, 7 mM β mercaptoethanol and 0.11% (v/v) Triton and was adjusted to pH 7.5 with 0.5 M HCl. EcoRI endonucleases (Miles Laboratories Ltd.,) had an activity of 3.8×10^4 units (mg protein) $^{-1}$ and was used undiluted. BamHI endonuclease with an activity of 9.0×10^4 units (mg protein) $^{-1}$ was also used undiluted. The mixture was incubated at 37°C for 1 h and the reaction stopped either by the addition of 10 μl 5% (w/v) SDS, 25% (v/v) glycerol plus 0.025% (w/v) bromophenol blue, followed by heating at 65°C for 10 min. or by the addition of 10 μl of a solution containing 7 M Urea, 50% (w/v) sucrose, 0.1 mM EDTA and 0.1% (w/v) bromophenol blue.

2.14.2 Agarose electrophoresis of endonuclease digested plasmid DNA.

The preparation of the agarose and the gel apparatus was the same as described in Section 2.13.2. The Tris borate electrophoresis buffer contained, g l^{-1} ; 10.8 Tris, 0.93 EDTA and 5.5 boric acid. 30 μl of the incubation mixture was applied to the agarose gel and run at room temperature at a constant voltage of 25 V (20 mA) for approximately 15 h or until the blue marker dye had almost reached the bottom of the gel. The gel was stained for 1 h in 0.5 μg

ethidium bromide ml^{-1} and photographed as described in Section 2.13.2. The logarithm of the migration distance of DNA molecular weight markers was plotted against the logarithm of molecular weight of the linear fragments to obtain a standard curve. The molecular weight of the plasmid DNA fragments of unknown size were calculated from the standard curve and expressed in $\times 10^6$ daltons.

2.15. DETERMINATION OF RATE OF PHOSPHATE ASSIMILATION

The rate of radioactive labeled $[^{32}\text{P}]$ -phosphorus assimilation was determined by the modified method of ^{K on references} Medveczy and Rosenberg (1971). 100 ml steady state culture was harvested by centrifugation at 23,000 g for 20 min. and the cell pellet resuspended in Hershey's defined medium (Section 2.3.3) lacking phosphate but with the normal concentration of glucose. The organisms were resuspended to give an absorbance (520 nm) equivalent to the growing chemostat culture before harvesting. An aliquot (normally 20 ml) was removed for dry weight (Section 2.8.3.) and viable count (Section 2.8.3.) analysis and the remaining suspension divided between two 100 ml Erlenmeyer flasks such that the first flask received 19.0 ml and the second flask 40 ml. The second flask was set aside at 37°C for 3 h with gentle shaking in order to starve the organisms. At the end of the 3 h starvation period 31 ml suspension was removed for biomass and viable count determination. The flasks were incubated at 37°C in a heated water bath. The flasks

were fitted with Suba seal vaccine stoppers and air bubbled through the culture via a Hamilton gauge 24 syringe needle, using a small air pump. Gas release was via a second syringe needle and tubing which led to a carbon dioxide trap. Samples were removed by a 1 ml disposable syringe fitted with a Hamilton gauge 24 syringe needle.

1 ml $[^{32}\text{P}]\text{-KH}_2\text{PO}_4$ with an activity of $0.02 \mu\text{Ci } \mu\text{mol}^{-1}$ was added to flask 1 to give a final $[^{32}\text{P}]\text{-phosphate}$ concentration of $2.5 \mu\text{moles phosphate ml}^{-1}$ with a specific activity of $0.05 \mu\text{Ci ml}^{-1}$. 1 ml samples were withdrawn at intervals throughout the first 30 min, filtered immediately through HAWP membrane filters (0.45μ pore size, 22mm diameter) and washed twice with 3 ml 140 mM NaCl solution 7 mM KH_2PO_4 . The membranes were soaked previously in the same solution before use to minimise adsorption of the $[^{32}\text{P}]\text{-label}$. The same method was adopted for the cell suspension in flask 2 except that the initial equilibration step was omitted. The rate of labelled phosphate uptake was expressed as nmol phosphate assimilated ($\text{mg dry weight}^{-1}$ hour $^{-1}$) or as nmol phosphate assimilated (1×10^9 organisms ml^{-1}) $^{-1}$ minute $^{-1}$.

2.16 COUNTING OF $[^{32}\text{P}]\text{-PHOSPHATE}$ RADIOACTIVITY

Radioactivity was determined by liquid scintillation counting using a Packard 2425 liquid scintillation counter. The membranes were placed in glass scintillation vials which received 10 ml scintillation fluid containing 500 ml Triton X - 100, 100 ^{ml/g?} sulphur-free toluene and 7 g, 2 - (4¹ tert -

butylphenyl) - 5 - 4 (4" - biphenyl) - 1 - 1, 3, 4 -
oxadiazole (Butyl BPD).

Standard vials with a known number of disintegrations per minute were used to check counting efficiency. The standard vials were prepared from a standard $[^{32}\text{P}]$ -orthophosphate solution in dilute HCl with a specific activity of $1 \mu\text{Ci ml}^{-1}$ (Table 2.2) and gave an average counting efficiency of 82%. This value correlated with an average external standard value of 80% which was the same as the values determined for the experimental vials. Accordingly it was assumed that the efficiency of counting was the same as the standard vials.

2.17 DETERMINATION OF RATE OF GLUCOSE ASSIMILATION

The rate of $[^{14}\text{C}]$ - glucose uptake was measured by the following method. The organisms were harvested as described in section 2.15. The organisms were resuspended in Davis and Mingioli medium (Section 2.3.2.) to give the same absorbance (520 nm) as in the chemostat culture. 18 or 19 ml of culture was distributed into 2 x 100 ml Erlenmeyer flasks as described in Section 2.16 and incubated at 37°C with aeration as described previously. One flask was used for $[^{14}\text{C}]$ - glucose uptake measurement while the second was monitored simultaneously for carbon dioxide release (Section 2.19). Uptake was measured under two conditions. In condition A unlabelled glucose was added to give a

Table 2.2. Efficiency of $[^{32}\text{P}]$ - phosphorus liquid scintillation counting.

$[^{32}\text{P}]$ - phosphorus 1 $\mu\text{Ci ml}^{-1}$ ml	Expected disintegrations dpm	Observed counts cpm	Efficiency of counting %	Automatic external standard value
0.05	1.11×10^5	95966	86	0.6586
0.025	5.55×10^4	42722	77	0.6830
0.01	2.22×10^4	18433	83	0.6990
0.005	1.11×10^4	8995.7	81	0.6996

concentration of 0.5 mg glucose ml⁻¹ (2.7 μmol glucose ml⁻¹), equilibrated for 10 min and then 50 μmoles of [¹⁴C] - glucose added to give a final concentration of 2.5 μmoles ml⁻¹ with a specific activity of 0.25 μCi ml⁻¹. 1.0 ml samples were taken at regular intervals over the first 60 min, filtered as previously described (Section 2.16) and washed with 3 ml ice-cold defined medium. The filters were transferred to glass scintillation vials and dried in a stream of air at room temperature. In condition B, 10 μmoles of [¹⁴C] - glucose was added to give a final concentration of 0.5 μmol glucose ml⁻¹ a specific activity of 0.1 μCi ml⁻¹.

The rate of glucose uptake was expressed as μmol of glucose assimilated (mg dry wgt)⁻¹ h⁻¹ or μmol of glucose assimilated (unit Absorbance)⁻¹ h⁻¹.

2.18 RADIORESPIROMETRY

The rate at which carbon dioxide was evolved was measured in parallel experiments with the uptake experiments (Section 2.18). Substrates were added simultaneously to the uptake and radiorespirometry flasks samples were taken by placing the syringe needle (Figure 2.9) into scintillation vials fitted with 8 ml carbon dioxide absorbing fluid containing 30% (v/v) 2 - aminoethanol in 2-methoxyethanol which had an absorbing capacity of 2.5 mM CO₂ ml⁻¹. Samples were taken at regular intervals

during the first 150 min. The rate was expressed as $\mu\text{mol carbon dioxide evolved (mg dry wgt)}^{-1} \text{ h}^{-1}$ or $\mu\text{mol carbon dioxide evolved (unit absorbance)}^{-1} \text{ h}^{-1}$.

¹⁴
2.) COUNTING $[^{14}\text{C}]$ - CARBON RADIOACTIVITY

Radioactivity was determined by liquid scintillation counting using a Packard 2425 liquid scintillation counter. For assimilation experiments (Section 2.18) vials were filled with 15 ml of a scintillation fluid containing 6.0 g 2 - (4¹-tert-butylphenyl)-5-4-(4" biphenyl)-1-1-3, 4- ~~oxadiazole~~ ^{oxadiazole} (Butyl - PBD) in 750 ml sulphur-free toluene and 250 ml methanol. For radiorespirometry (Section 2.18) 12 ml of a scintillant containing 0.02% (w/v) 1, 4- BIS (2-5 phenyl oxadyl) benzene (POPOP), 0.4% (w/v) 2, 5 diphenyl oxazole (PPO) in a solvent of 3 volumes of toluene plus 2 volumes of 2- ethoxyethanol (3 : 2 v/v).

Vials with a known number of disintegrations per minute were used at regular intervals to check the counting efficiency. Standard vials were prepared from a standard n - hexadecane -1 $[^{14}\text{C}]$ solution with an activity of $1.1 \mu\text{Ci g}^{-1}$. The average efficiency of counting was 78%. The automatic external standard gave the same values and it was assumed that the efficiency of counting was the same as in standard vials.

Table 2.3. Efficiency of $[^{14}\text{C}]$ - glucose liquid scintillation counting.

n-Hexadecane 1.1 $\mu\text{Ci g}^{-1}$ g	Expected disintegrations dpm	Observed counts cpm	Efficiency %	Automatic external standard value
0.10095	244249	190936	78.15	0.5603
0.0706	170852	140231	82.0	0.5663
0.00398	96315	7380	76.6	0.5464
0.00151	3654	2810	76.9	0.5606

3. THE STABILITY OF PLASMID TP120 IN POPULATIONS OF
ESCHERICHIA COLI K12 STRAIN IR713 IN CONTINUOUS
FLOW CULTURE.

3.1. DETERMINATION OF FREQUENCY OF PLASMID MARKER
LOSS IN CLOSED CULTURE

E. coli K12(TP120) was grown overnight in closed culture in complex and defined drug-free media to determine the frequency of spontaneous drug sensitive mutants in non-limiting medium.

Table 3.1. shows that no ampicillin, streptomycin or sulphonamide sensitive organisms were detected in 2×10^3 E. coli K12(TP120) organisms. In contrast one tetracycline sensitive organism appeared in 1×10^3 organisms during overnight growth. The frequency of loss of the drug resistance marker was the same in the three growth media (Table 3. 1.)

3.2. CARBON-LIMITED CHEMOSTAT SELECTION EXPERIMENTS

The closed culture experiments indicated that in populations of Escherichia coli carrying plasmid TP120, the plasmid was retained but with some indication of a modification of the drug resistance pattern, namely the measurable, low level of tetracycline resistance loss. As indicated in the Introduction (Section 1.7) closed culture growth conditions probably

Table 3.1 Frequency of loss of resistance markers in E. coli K12 IR713(TP120)
in closed culture in complex and defined media.

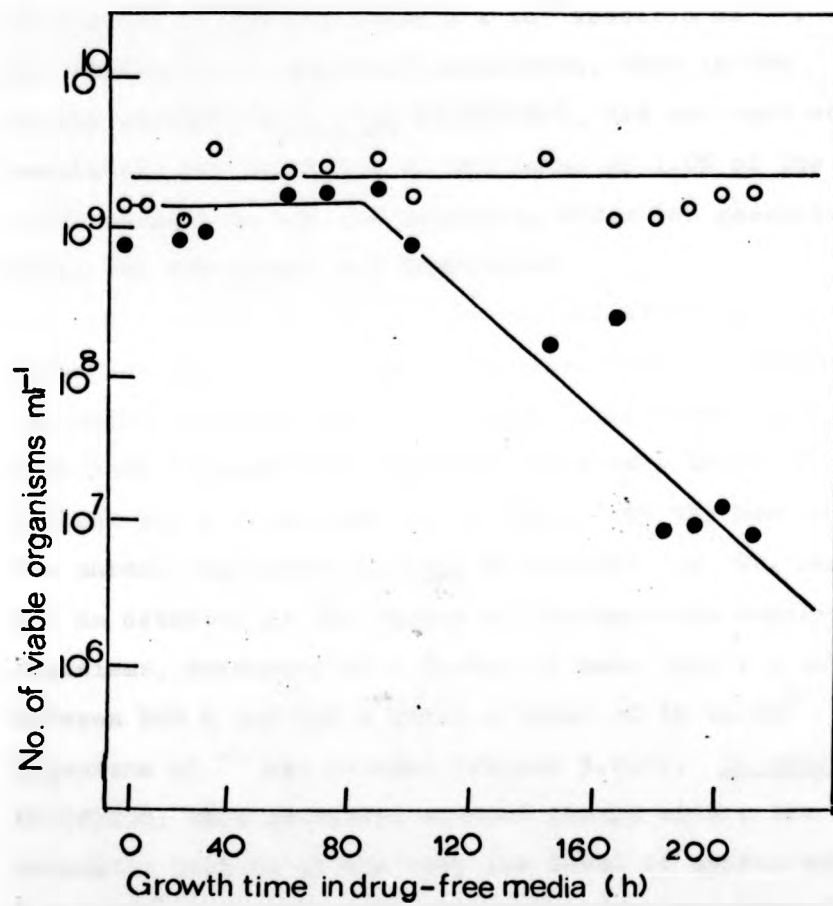
Drug resistance marker	Frequency of marker loss (2×10^3 cells) -1		
	Nutrient Broth	Davis & Mingioli medium	Hershey medium.
Ap.	0	0	0
Sm	0	0	0
Su	0	0	0
Tc	2	1	2

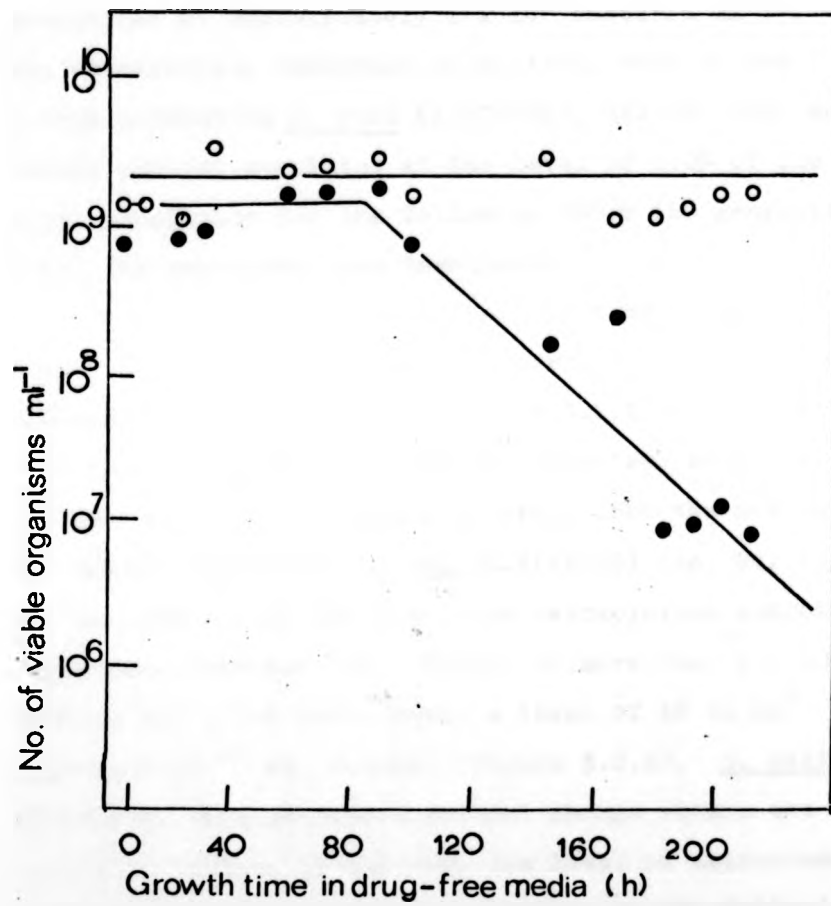
do not apply strong selective conditions against the maintenance of the plasmid within the population. Accordingly, the stability of plasmid TP120, or otherwise, was examined in drug-free nutrient-limited conditions at sub-maximal growth rates in chemostat culture.

Initially plasmid persistence was investigated in growth conditions limited by the availability of carbon required for energy and biosynthesis at the low dilution rate of 0.10 h^{-1} . The plasmid-carrying organisms E. coli K12(TP120) were plated out onto drug containing agar plates and loss of a drug resistance marker detected by a decrease in the number of colonies growing on these plates (Section 2.9).

In two experiments at a dilution rate of 0.10 h^{-1} tetracycline resistance was shown to be the most unstable marker as predicted from closed culture observations. In the initial experiment (Selection experiment number 1.) E. coli K12(TP120) persisted in the chemostat in drug-free carbon-limited medium for approximately 80 h (Figure 3.1). After this period of growth the number of tetracycline resistant colonies on agar plates began to decrease showing loss of this marker from the population at a rate of $- 0.04 \text{ h}^{-1}$ (Table 3.3) until a level of 1×10^7 tetracycline

Figure 3.1 The growth of E. coli K12(TP120) in a carbon-limited chemostat at $D = 0.10 \text{ h}^{-1}$ showing loss of the tetracycline resistance marker (selection experiment number 1). Total number of viable organisms (○) and number of organisms resistant to tetracycline (●).





resistant bacteria ml^{-1} was reached. During this period the remainder of the drug resistance markers were maintained at approximately 1×10^9 bacteria ml^{-1} . The tetracycline resistant population, that is the parent population E. coli K12(TP120), did not wash out completely but persisted at the level of 1.0% of the total population for the following 200 h (80 generations) until the experiment was terminated.

In a repeat experiment (selection experiment number 2) growth in a drug-free carbon-limited medium was maintained for 1800 h. Figure 3.2.1 shows that the number of tetracycline resistant organisms began to decline 500 h after removal of drugs from the medium. The parent population E. coli K12(TP120) (Ap, Sm, Su, Tc) as detected by the number of tetracycline resistant organisms, decreased by a factor of more than 1×10^6 between 500 h and 800 h until a level of 10 to 10^2 organisms ml^{-1} was reached (Figure 3.2.1). E. coli K12(TP120) then persisted without change within the chemostat culture at the very low level of approximately 5.0×10^{-5} % of the total population for the following 1000 h (144 generations) (Table 3.2). The remaining drug resistance markers declined slightly but were maintained at between 5×10^8 to 1×10^9 bacteria ml^{-1} (Figure 3.2.2.). The new dominant population E. coli

Figure 3.2.1. The growth of *E. coli* K12(TP120) in a carbon-limited chemostat at $D = 0.1 \text{ h}^{-1}$ showing loss of the tetracycline resistance marker (selection experiment number 2). Total number of viable organisms (O) and number of organisms resistant to tetracycline (●).

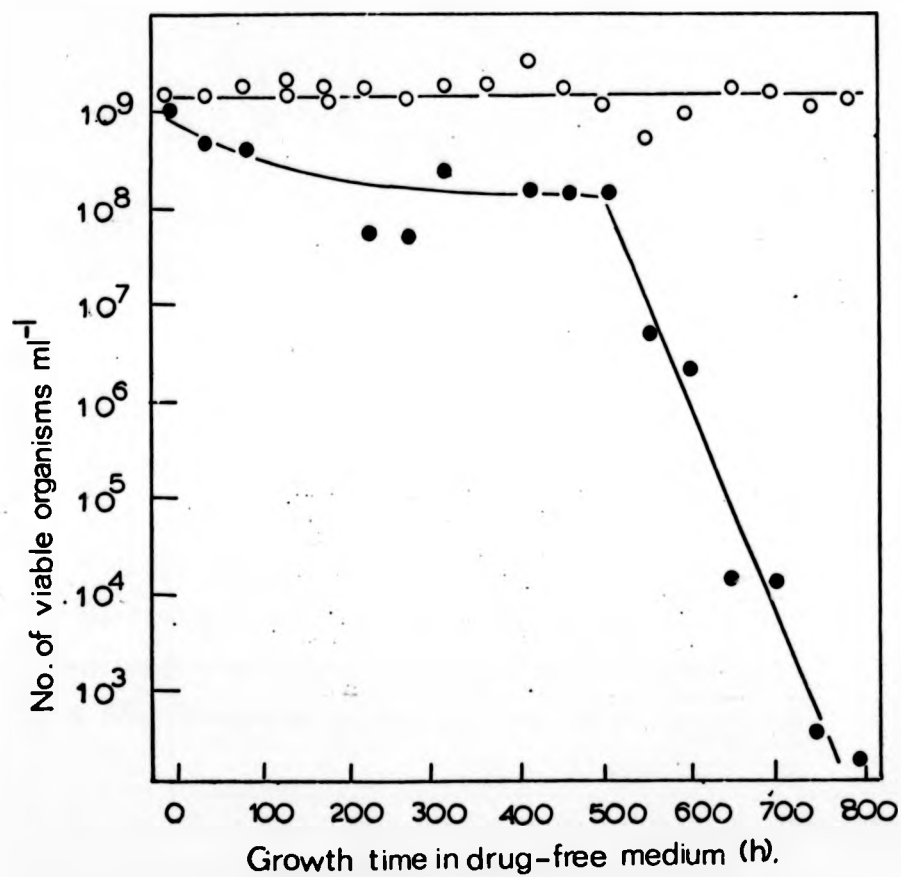


Figure 3.2.2. The growth of E. coli K12(TP120) in a carbon-limited chemostat at $D = 0.10 \text{ h}^{-1}$ showing maintenance of ampicillin, streptomycin and sulphonamide resistance markers (selection experiment number 2). Total number of organisms resistant to ampicillin (\circ); streptomycin (\bullet); and sulphonamide (Δ).

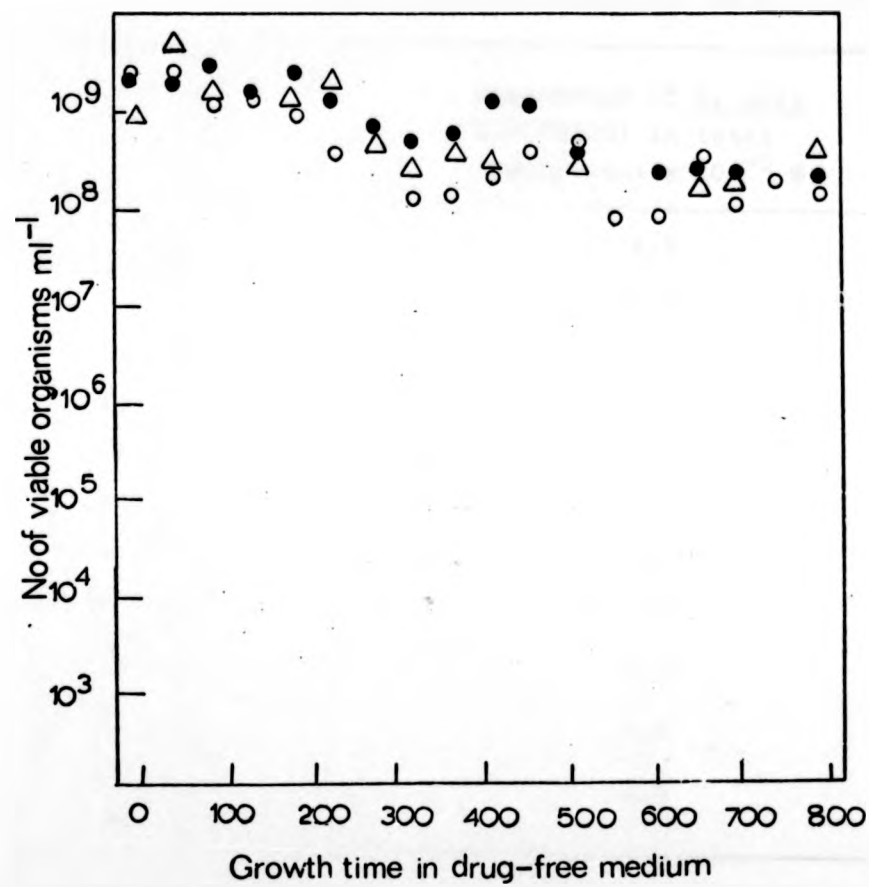


Table 3.2. Persistence of parent strain E. coli K12(TP120) for 900 h following washout in carbon-limited chemostat selection experiment at $D = 0.1 \text{ h}^{-1}$

Time (h)	Percentage of <u>E. coli</u> K12(TP120) in total population: $\times 10^{-5} \%$
800	2.3
900	21.9
1000	24.0
1100	6.2
1200	13.0
1300	7.8
1400	9.2
1500	5.6
1600	3.8
1700	6.3

K12(TP120A) (Ap, Sm, Su) which had successfully replaced the tetracycline resistant E. coli K12(TP120) (Ap, Sm, Su, Tc) was later shown to have retained a plasmid (Table 3.5)

3.3 PHOSPHOROUS-LIMITED CHEMOSTAT SELECTION EXPERIMENTS

The growth of the plasmid-carrying strain E. coli K12(TP120) was also examined in phosphorous-limited conditions at constant dilution rates of 0.10 h^{-1} and 0.35 h^{-1} . It was speculated that in these conditions the limited amount of available phosphate would be utilised preferentially for essential chromosomal DNA synthesis and associated protein synthesis, thereby establishing strong selective conditions against the maintenance of a plasmid-containing population since these organisms would be engaged in the unnecessary synthesis of DNA coding for non-essential functions, namely drug resistance.

In a preliminary experiment at $D = 0.10 \text{ h}^{-1}$, in drug-free phosphorous-limited conditions (Selection experiment number 3) (Figures 3.3.1 and 3.3.2) the number of ampicillin and streptomycin resistant organisms declined to levels of approximately 1×10^4 bacteria ml^{-1} while the total number of organisms, as assayed as drug-

Figure 3.3.1. The growth of E. coli K12(TP120) in a phosphate-limited chemostat at $D = 0.10 \text{ h}^{-1}$ showing loss of the tetracycline and ampicillin resistance markers (selection experiment number 3). Total number of organisms (\circ) and number of organisms resistant to tetracycline (\bullet) and ampicillin (Δ).

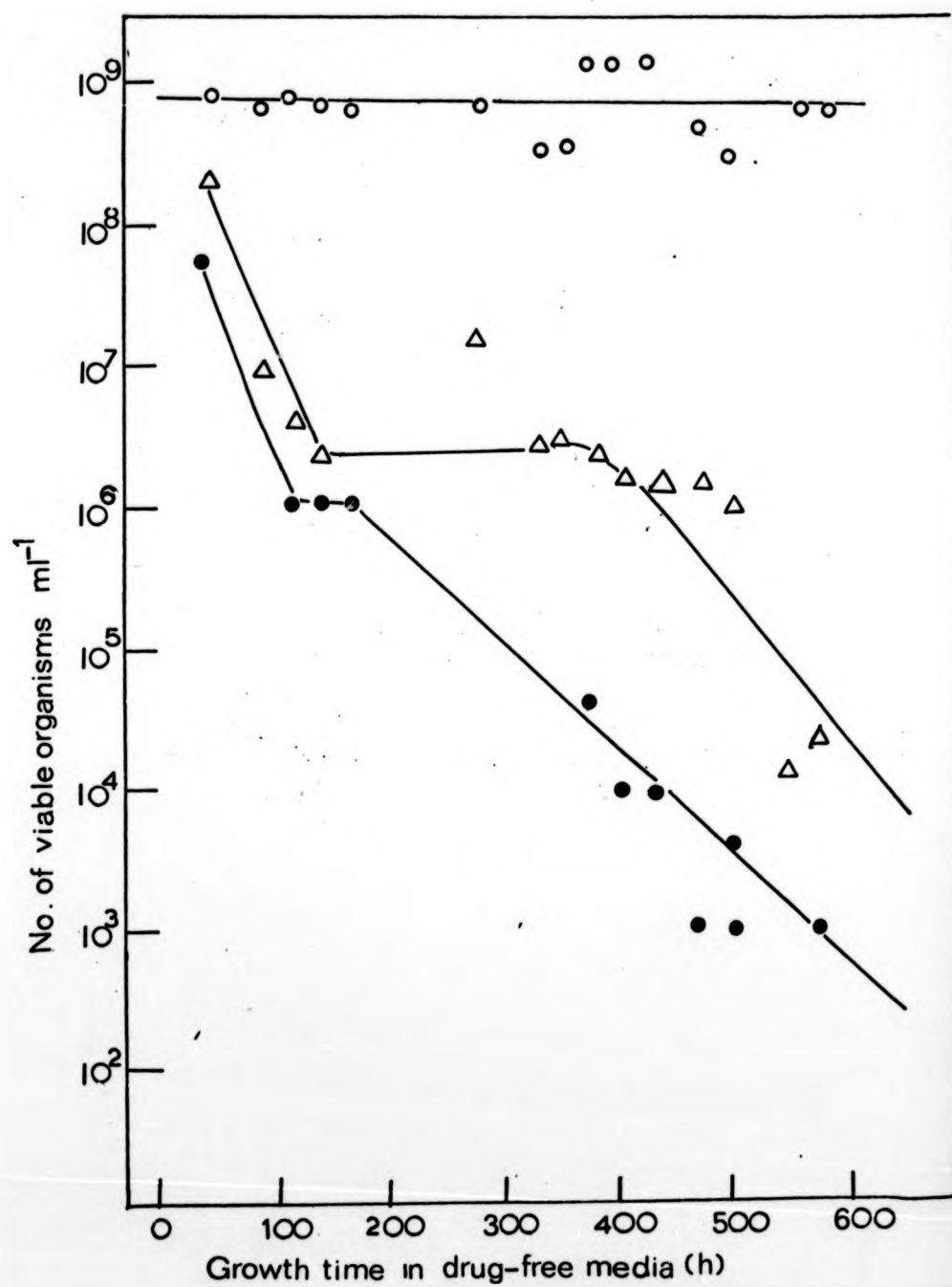
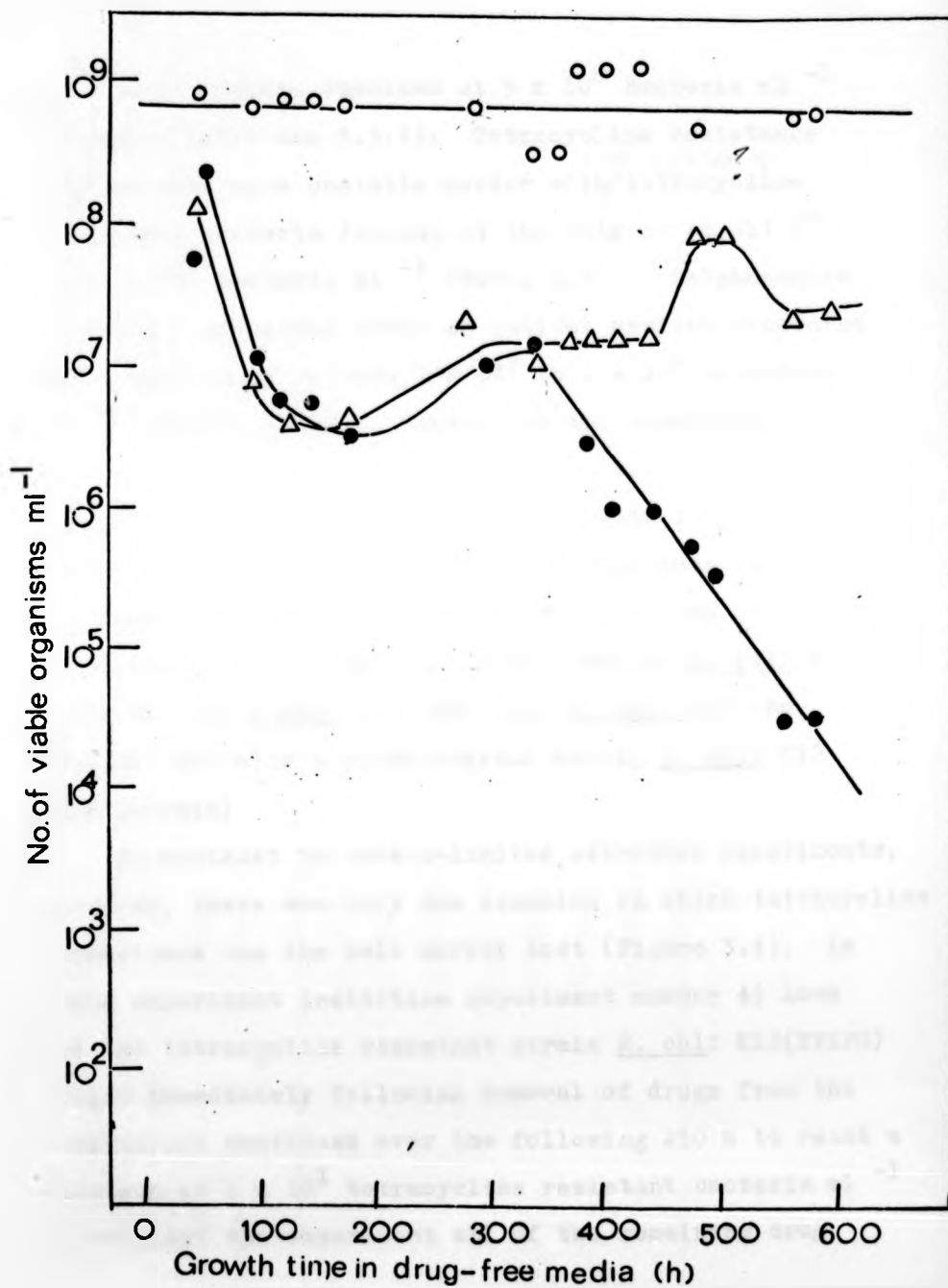


Figure 3.3.2. The growth of E. coli K12(TP120) in a phosphate-limited chemostat at $D = 0.10 \text{ h}^{-1}$ showing loss of the streptomycin resistance marker (selection experiment number 3). Total number of organisms (○) and number of organisms resistant to streptomycin (●) and sulphonamide (4).

1 7



free agar plates, remained at 5×10^8 bacteria ml⁻¹ (Figures 3.3.1 and 3.3.2). Tetracycline resistance was an even more unstable marker with tetracycline resistant bacteria falling at the rate of -0.017 h^{-1} to 1×10^3 bacteria ml⁻¹ (Table 3.3). Sulphonamide resistant organisms after an initial decline recovered and remained at between 1×10^7 to 1×10^8 organisms ml⁻¹ (Figure 3.3.2). Ampicillin and streptomycin resistant organisms were lost at rates of approximately -0.023 h^{-1} (Table 3.3) over 400 h growth in drug free medium. At the termination of the experiment a number of organisms carrying different combinations of drug resistance markers were isolated, namely, E. coli K12 (Ap, Sm, Su) E. coli K12 (Sm, Su), E. coli K12 (Sm, Su, Tc) and also a plasmid-minus strain E. coli K12 (no plasmid)

In contrast to carbon-limited selection experiments, however, there was only one occasion on which tetracycline resistance was the sole marker lost (Figure 3.4). In this experiment (selection experiment number 4) loss of the tetracycline resistant strain E. coli K12(TP120) began immediately following removal of drugs from the medium and continued over the following 250 h to reach a minimum of 1×10^1 tetracycline resistant bacteria ml⁻¹. Throughout the experiment all of the remaining drug

Table 3.3 Rate of drug resistance marker loss and growth rates of the uncompetitive populations in chemostat selection experiments.

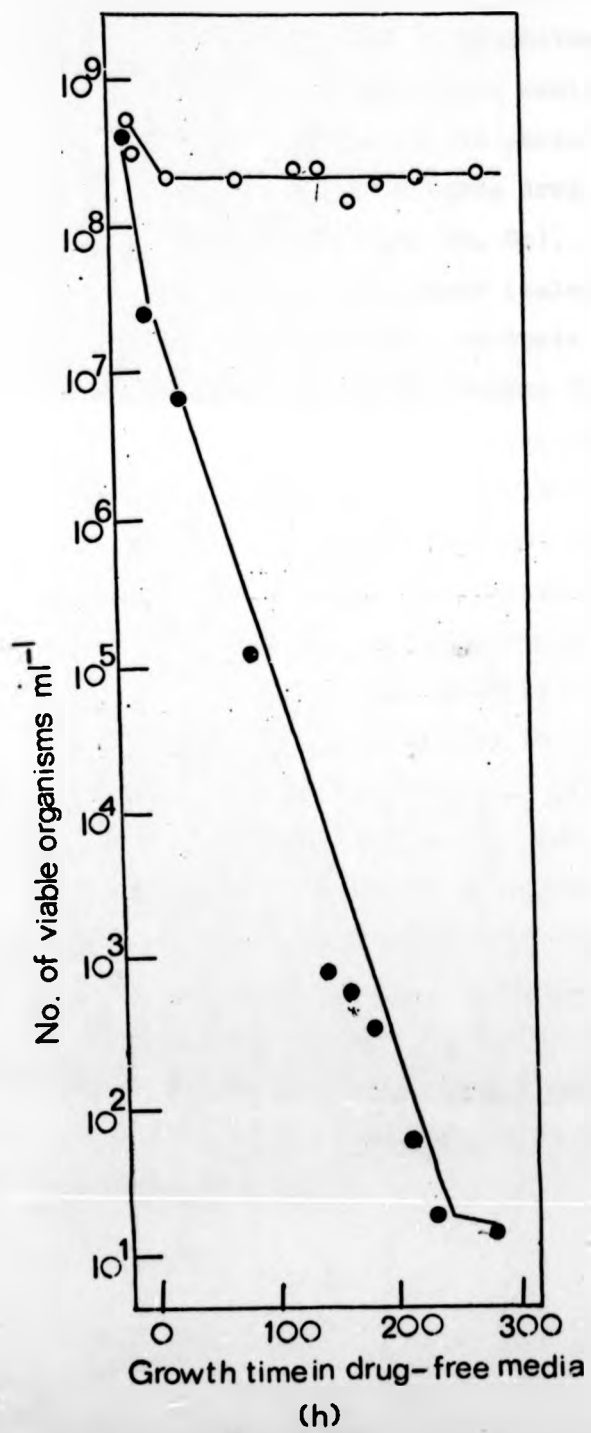
a = Growth rate μ_1 of uncompetitive population calculated from $\mu_1 = \frac{\ln x_t - \ln x_0}{(t_2 - t_1)} + D$ (Section 1)

Selection Experiment	Nutrient Limitation	Dilution Rate (h ⁻¹)	Rate of marker loss (h ⁻¹)		Growth rate, μ_1 , of uncompetitive population <u>E. coli</u> K12(TP120) ^a (h ⁻¹)
			Tc	Ap	
1	Carbon	0.1	-0.04	0	0.06
2	Carbon	0.1	-0.055	0	0.045
3	Phosphate	0.1	-0.017	-0.023	0.08
4	Phosphate	0.1	-0.07	0	0.03
5	phosphate	0.1	-0.027	-0.029	0.07
6	Phosphate	0.1	-0.34	-0.29	0
7	Phosphate	0.35	-0.012	0	0.33

Negative signs incorrect

X

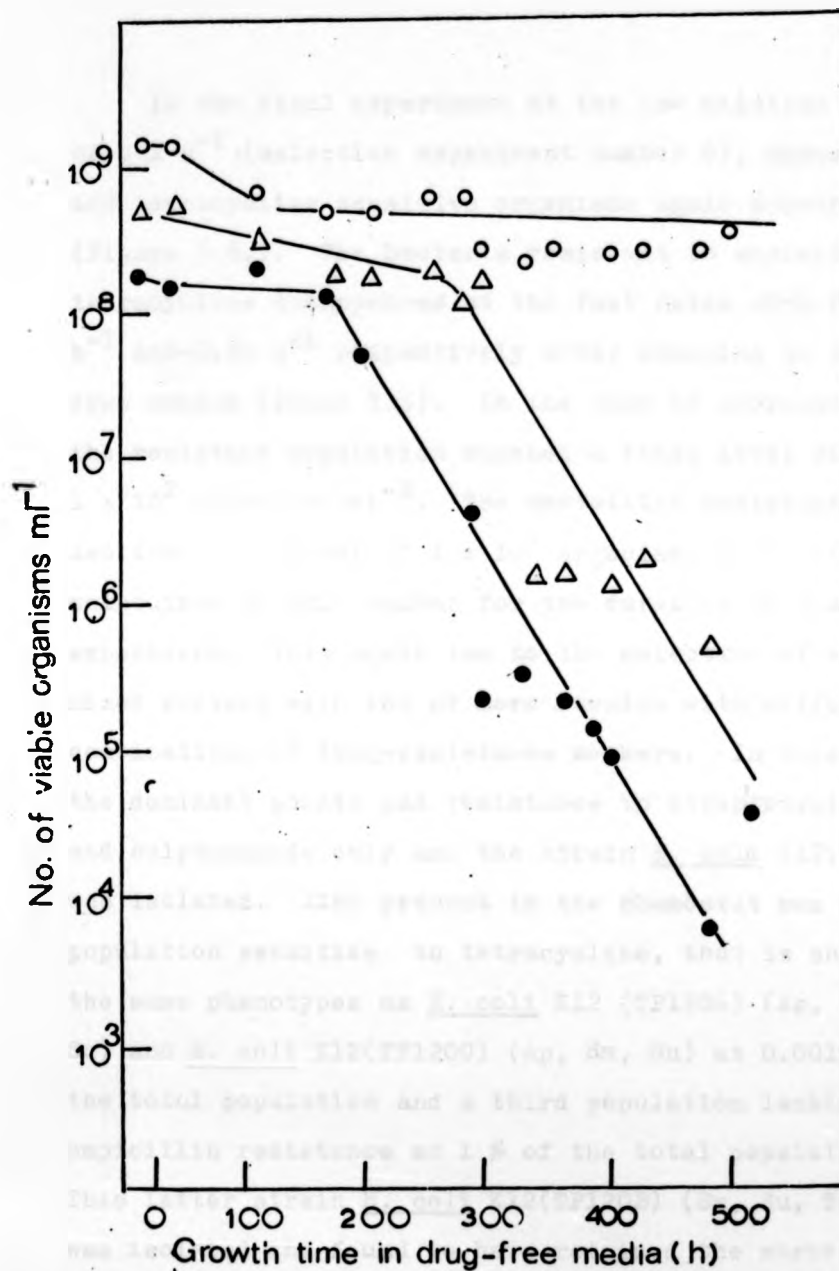
Figure 3.4 The growth of E. coli K12(TF120) in a phosphate-limited chemostat at $D = 0.10 \text{ h}^{-1}$ showing loss of the tetracycline resistance marker (selection experiment number 4). Total number of organisms (O) and organisms resistant to tetracycline (●).



resistances were present at levels above 1×10^8 resistant organisms ml^{-1} demonstrating that the disappearance of tetracycline resistant E. coli K12(TP120) reflected the washout of the parent population and the and the domination of a three drug resistant strain E. coli K12(TP120C) (Ap, Sm, Su).

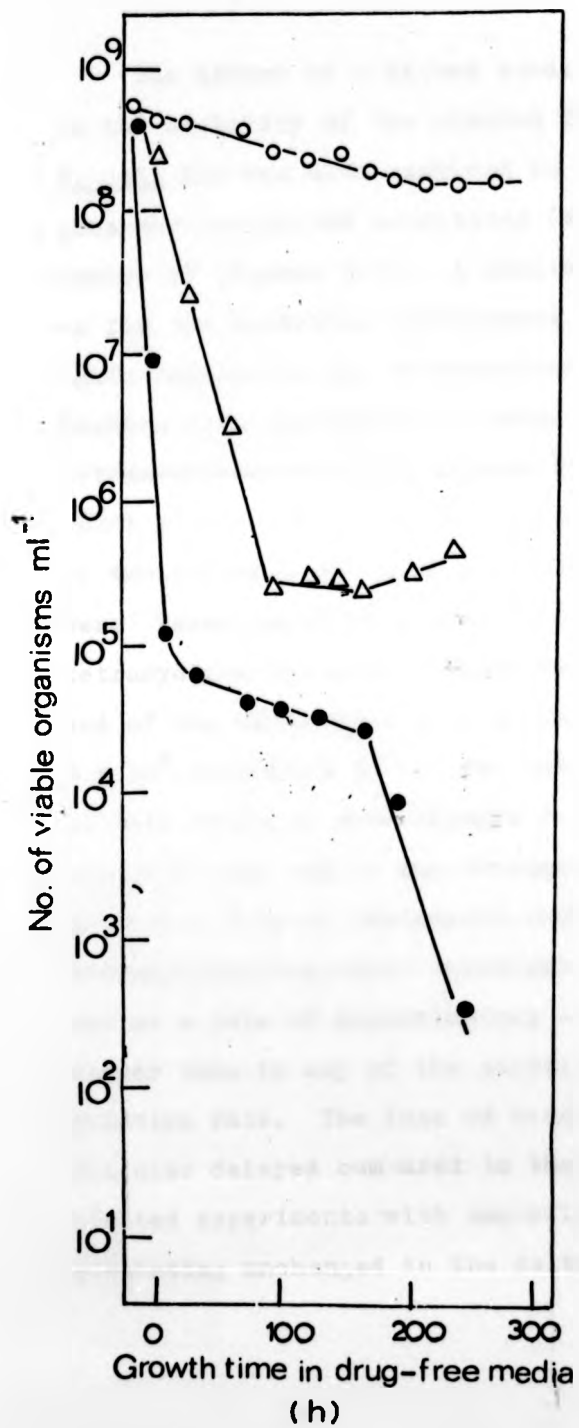
In a further experiment (selection experiment number 5) tetracycline resistant bacteria were again lost from the chemostat population (Figure 3.5). This experiment also showed that ampicillin resistance was another marker frequently lost in phosphorus-limited selection experiments. The ampicillin and tetracycline resistant organisms disappeared from the population at similar rates of -0.027 h^{-1} for tetracycline resistance and -0.029 h^{-1} for ampicillin resistance (Table 3.3). The streptomycin and sulphonamide resistant organisms persisted at levels between 1×10^8 to 1×10^9 bacteria ml^{-1} for the duration of the experiment. The number of tetracycline resistant organisms fell to 1×10^4 organisms ml^{-1} and ampicillin resistant bacteria to 1×10^5 organisms ml^{-1} . Organisms with resistances to only ampicillin, streptomycin and sulphonamide and only streptomycin, sulphonamide and tetracycline were isolated but were not selected for further study or characterisation.

Figure 3.5. The growth of E. coli K12(TP120) in a phosphate-limited chemostat at $D = 0.10 \text{ h}^{-1}$ showing loss of tetracycline and ampicillin resistance markers (selection experiment number 5). Total number of organisms (o) and number of organisms resistant to tetracycline (●) and ampicillin (Δ).



In the final experiment at the low dilution rate of 0.1 h^{-1} (selection experiment number 6), ampicillin and tetracycline sensitive organisms again appeared (Figure 3.6.). The bacteria resistant to ampicillin and tetracycline disappeared at the fast rates of -0.29 h^{-1} and -0.34 h^{-1} respectively after changing to the drug-free medium (Table 3.3). In the case of tetracycline, the resistant population reached a final level of 1×10^2 organisms ml^{-1} . The ampicillin resistant organisms declined to a level of 1×10^5 organisms ml^{-1} and were maintained at this number for the duration of the experiment. This again led to the selection of a mixed culture with two or more strains with different combinations of drug-resistance markers. In this case the dominant strain had resistance to streptomycin and sulphonamide only and the strain E. coli K12(TP120D) was isolated. Also present in the chemostat was a population sensitive to tetracycline, that is showing the same phenotypes as E. coli K12 (TP120A) (Ap, Sm, Su) and E. coli K12(TP120C) (Ap, Sm, Su) at 0.001% of the total population and a third population lacking ampicillin resistance at 1 % of the total population. This latter strain E. coli K12(TP120B) (Sm, Su, Tc) was isolated and found to have retained the markers for streptomycin, sulphonamide and tetracycline resistance.

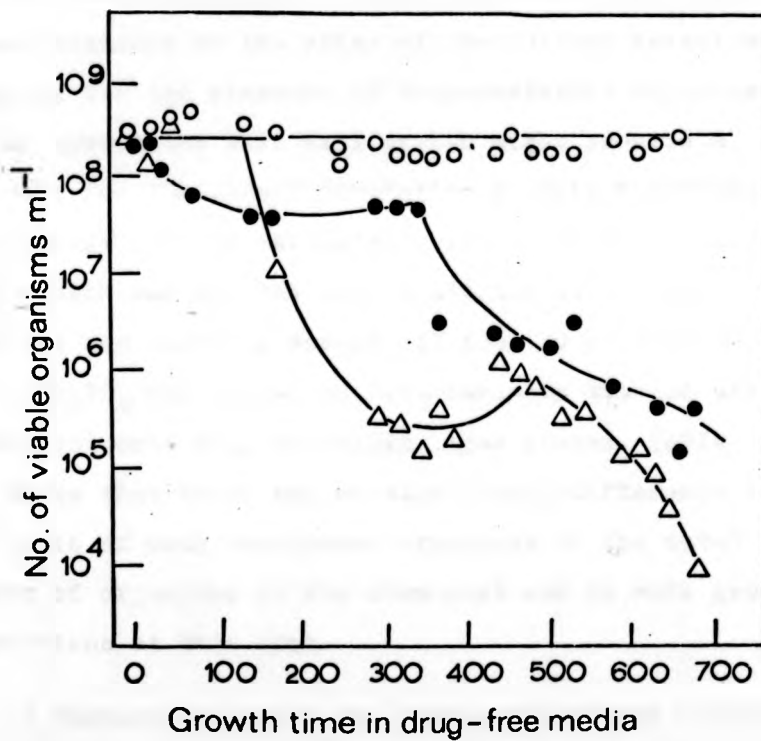
Figure 3.6. The growth of E. coli K12(TP120) in a phosphate-limited chemostat at $D = 0.10 \text{ h}^{-1}$ showing loss of tetracycline and ampicillin resistance markers (selection experiment number 6). Total number of organisms (\circ) and number of organisms resistant to tetracycline (\bullet) and ampicillin (Δ).



The affect of a higher rate; $D = 0.35 \text{ h}^{-1}$, on the stability of the plasmid TP120 in populations of E. coli K12 was also examined in a single experiment under phosphorus-limited conditions (selection experiment number 7) (Figure 3.7). A similar result was obtained as for the selection experiments at $D = 0.10 \text{ h}^{-1}$. Again ampicillin and tetracycline were the least stable markers with the number of colonies growing on tetracycline-containing plates falling from a viable count of 2×10^8 to 1×10^5 organisms ml^{-1} . In contrast to the previous phosphorus-limited experiments there were fewer ampicillin resistant organisms than tetracycline resistant organisms in the population at the end of the experiment i.e. a final plate count of 8×10^3 organisms ml^{-1} . The loss of resistance markers at this dilution rate (Figure 3.7) did not follow linear slopes and it was consequently difficult to measure a rate of resistance marker loss (Table 3.3). Tetracycline resistant organisms were eventually washing out at a rate of approximately -0.012 h^{-1} which was slower than in any of the experiments at the lower dilution rate. The loss of drug resistant organisms was also delayed compared to the previous phosphorus-limited experiments with ampicillin resistant organisms persisting unchanged in the culture for 200 h growth in

Figure 3.7. The growth of E. coli K12(TP120) in a phosphate-limited chemostat at $D = 0.35 \text{ h}^{-1}$ showing loss of tetracycline and ampicillin resistance markers (selection experiment number 7). Total number of organisms (O) and organisms resistant to tetracycline (●) and ampicillin (Δ).

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drug-free medium (Figure 3.7). Tetracycline resistance declined by a factor of 10 during the first 350 h of growth but then the number fell from 5×10^7 to 1×10^5 bacteria ml^{-1} over the following 350 h.

At the termination of the experiment the bacterial growth attached to the sides of the culture vessel was examined for the presence of drug-resistant organisms. It was speculated that wall growth might provide a reservoir for the parent population E. coli K12(TP120) and account for its persistence in the culture. The wall growth was removed with a sterile cotton wool swab and the bacteria washed off into 10 ml sterile 0.1 M $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer pH 7.0, serially diluted and plated out onto drug containing agar plates. Table 3.4 shows that there was no significant difference in the ratio of drug resistance organisms to the total number of organisms in the chemostat and in wall growth populations at this time.

A feature common to two phosphate-limited selection experiments, $D = 0.10 \text{ h}^{-1}$ (Figures 3.3 and 3.6) and the one at the higher dilution rate $D = 0.35 \text{ h}^{-1}$ (Figure 3.7) was that the slopes of the loss of individual drug resistance markers were not constant and therefore did not follow the anticipated washout kinetics. Theoretical

Table 3.4. Comparison of the number of drug resistant organisms contained in chemostat culture and in wall growth of a culture vessel. (Percentage of resistant organisms in the total population.) Viable counts were determined on nutrient agar plates containing antibiotics at 60% of MIC values.

<u>E. coli</u> K12 (TPI20)	Viable count of bacteria ml ⁻¹				
	Total Count	Ap	Sm	Su	Tc
Chemostat culture	2.0×10^8	3.6×10^4 (0.08)	1.39×10^8 (69.0)	1.4×10^8 (70.0)	4.3×10^3 (0.002)
Wall growth	2.07×10^8	2.03×10^3 (0.00)	8.5×10^6 (42.5)	8.0×10^6 (40.0)	1.0×10^3 (0.005)

kinetics predicted the disappearance of a population from the chemostat at a constant rate (Section 1.7.8). The slopes shown in Figures 3.3., 3.6 and 3.7 demonstrated that the resistant organisms were apparently lost in a non-linear fashion from the chemostat, exhibiting plateau periods and occasionally short phases of transient increases in the number of drug resistant organisms before washout continued.

Washout kinetics (Section 1.7.6) also predicted than an unsuccessful, uncompetitive population, such as E. coli K12(TP120) would eventually be eliminated from the continuous-flow culture. However in phosphorus limited chemostats this was never demonstrated and instead, as in carbon-limited condition, the parent population E. coli K12(TP120) after an initial period of decline persisted in the chemostat population at levels between 0.001 - 1% of the total bacterial population (Figures 3.3 to 3.7).

Compared with carbon-limited stability studies a more complex pattern of drug resistance marker loss associated with an apparent increase in parent strain plasmid instability was observed for phosphorus-limited selection experiments. In common with selection in carbon-limited conditions, the tetracycline resistance

marker was the most unstable since it was routinely lost from steady state culture at varying rates (Table 3.3) in each experiment. The loss of the resistance markers also began sooner after the change to drug-free phosphorus-limited medium compared to carbon-limited selection (Figure 3.2) where the plasmid-carrying E. coli K12(TP120) was maintained for 500 h in the chemostat before tetracycline resistance was lost.

As a result of the continuous-flow selection procedures a number of different strains with different combinations of drug resistance markers were isolated but only five representative strains were chosen for further characterisation. Four of these strains were shown by caesium chloride centrifugation and agarose electrophoresis (Section 7) to carry plasmids and were therefore termed E. coli K12(TP120A) (Ap, Sm, Su) (isolated from selection experiment 1); E. coli K12(TP120B) (Sm, Su, Tc) (from selection experiment 6); E. coli K12(TP120C) (Ap, Sm, Su) (from selection experiment 4) and E. coli K12(TP120D) (Sm, Su) (isolated from selection experiment 6). The remaining strain was the plasmid-less E. coli K12 evolved in the initial phosphorus-limited chemostat selection experiment number 3 and called E. coli K12(TP120E) (no plasmid).

3.4 TRANSFER OF THE ORIGINAL PLASMID TP120 AND AND EVOLVED PLASMIDS INTO E. COLI K12 711 NaI^R

The strains evolved in the carbon and phosphorus limited chemostat selection experiments were tested for their ability to transfer their drug resistance to a plasmid-free host and the frequency of transfer, if any, compared to that shown for the parent strain E. coli K12(TP120) and the same recipient.

In preliminary mating experiments in liquid nutrient broth it was not possible to demonstrate the transfer of any of the drug plasmids into the nalidixic acid recipient E. coli K12 711 NaI^R.

However the transfer ability, or otherwise, of the plasmid TP120 and the plasmids of the evolved strains was demonstrated in conjugation experiments carried out on solid media and the rates of transfer were measured for complete and defined media (Table 3.5). It was shown that two of the evolved strains E. coli K12(TP120C) and E. coli K12(TP120D) were unable to transfer their drug resistance markers (Table 3.5). For the remaining evolved strains E. coli K12(TP120A) and E. coli K12(TP120B), whilst retaining their ability to transfer plasmid DNA into the plasmid-minus recipient E. coli K12 711 NaI^R, the frequency of transfer was reduced compared with E. coli K12(TP120) (Table 3.5). The difference was particularly marked in Davis and Mingioli defined medium

Table 3.5 Presence of plasmids in selected E. coli K12 Strains and their frequencies of transfer to E. coli.

Strains were derived from continuous-flow selection experiments at a dilution rate of 0.10 h⁻¹ under carbon-limited conditions (a) or phosphor us-limited conditions (b). Transfer frequencies are the mean of 5 separate experiments and \pm indicated standard deviation values.

<u>E. coli</u> K12 with plasmid (resistance markers)	Presence of plasmid in <u>E. coli</u> K12	Percentage frequency of marker transfer $\times 10^{-3}$		
		Nutrient Droth.	Davis & Mingioli defined medium.	Hershey defined medium.
TP120 (Ap, Sm, Su, Tc)	+	5.5 \pm 0.7	5.3 \pm 0.6	1.8 \pm 0.3
TP120A ^a (Ap, Sm, Su)	+	5.0 \pm 0.5	0.1 \pm 0.03	0.3 \pm 0.02
TP120B ^b (Sm, Su, Tc)	+	1.4 \pm 0.2	0.1 \pm 0.05	0.6 \pm 0.03
TP120C ^b (Ap, Sm, Su)	+	0	0	0
TP120D ^b (Sm, Su)	+	0	0	0

where the percentage frequency transfer of markers was reduced from 5.0×10^{-3} to 0.1×10^{-3} for the evolved strains. The transfer frequencies were higher for all the strains in the undefined medium. X

3.5 CLOSED CULTURE MEASUREMENT OF THE MAXIMUM SPECIFIC GROWTH RATES OF THE PARENT AND EVOLVED STRAIN OF E. COLI K12.

The continuous-flow culture stability experiments showed that under nutrient-limited conditions it was possible to select for mutant strains carrying a decreased number of drug resistance markers. In prolonged selection experiments the derivative strains that were resistant to three or more drugs tended to dominate the original organism suggesting that under the prevailing growth conditions these strains had a selective growth advantage. Any possible advantage in increase of growth rate, specifically the maximum specific growth rate, was therefore examined in closed culture in complete and defined media.

Table 3.6 shows that the effect of the loss of expression of drug resistance markers on the growth rate of the evolved strains of E. coli K12 in nutrient broth was variable. In this medium there was little difference in μ_{\max} values of the parent strain E. coli K12(TP120) and the evolved strains E. coli K12(TP120A)

Table 3.6. Maximum specific growth rates of selected E. coli K12 strains carrying various drug resistance markers, or none, in closed culture in nutrient broth.

Strains had either never been grown in continuous-flow culture (a) or were isolated from chemostat culture experiments at $D = 0.10 \text{ h}^{-1}$ under carbon-limited (b) or phosphorous-limited conditions (c)

<u>E. coli</u> K12 with plasmid (resistance markers)	μ_{max} (h^{-1})	Percentage increase of evolved strain μ_{max} compared with <u>E. coli</u> K12(TP120)
TP120 ^a (Ap, Sm, Su, Tc)	0.53	-
TP120A ^b (Ap, Sm, Su)	0.54	2
TP120B ^c (Sm, Su, Tc)	0.74	40
TP120C ^c (Ap, Sm, Su)	0.55	4
TP120D ^c (Sm, Su)	0.75	43
TP120E ^c (no plasmid)	0.59	11
IR713 ^a (no plasmid)	0.54	2

E. coli K12(TP120C) and the plasmid-minus strain E. coli K12(TP120E)(no plasmid). Furthermore the isogenic plasmid-minus strain E. coli K12 IR713 which had not been grown in chemostat culture also had a similar μ_{\max} value under these growth conditions. The parent strain E. coli K12(TP120) had the lowest maximum specific growth rate of 0.53 h^{-1} whilst the evolved strains already mentioned had μ_{\max} values not significantly greater at 2 to 4% higher than the parent strain. However E. coli K12(TP120B) (Sm, Su, Tc) and E. coli K12(TP120D)(Sm, Su) has maximum specific growth rates which were approximately 40% higher than the maximum specific growth rate of E. coli K12(TP120) (Ap, Sm, Su, Tc).

In the defined Davis and Mingioli medium the parent strain E. coli K12(TP120) again had the lowest μ_{\max} value which under these conditions was 0.40 h^{-1} . (Table 3.7 and Figure 3.8.1.). However, the four mutant strains carrying plasmids coding for resistance to two or three drugs were all capable of faster maximum specific growth rates (Table 3.7, Figures 3.8.1 and 3.8.2). In defined medium the tetracycline sensitive strains E. coli K12 (TP120A) and E. coli K12(TP120C) had growth rates of 0.56 h^{-1} and 0.57 h^{-1} respectively, which were 40 to 43% greater than the

Table 3.7. Maximum specific growth rates of selected *E. coli* K12 strains carrying various drug resistance markers, or none, in closed culture in Davis and Mingioli defined medium.

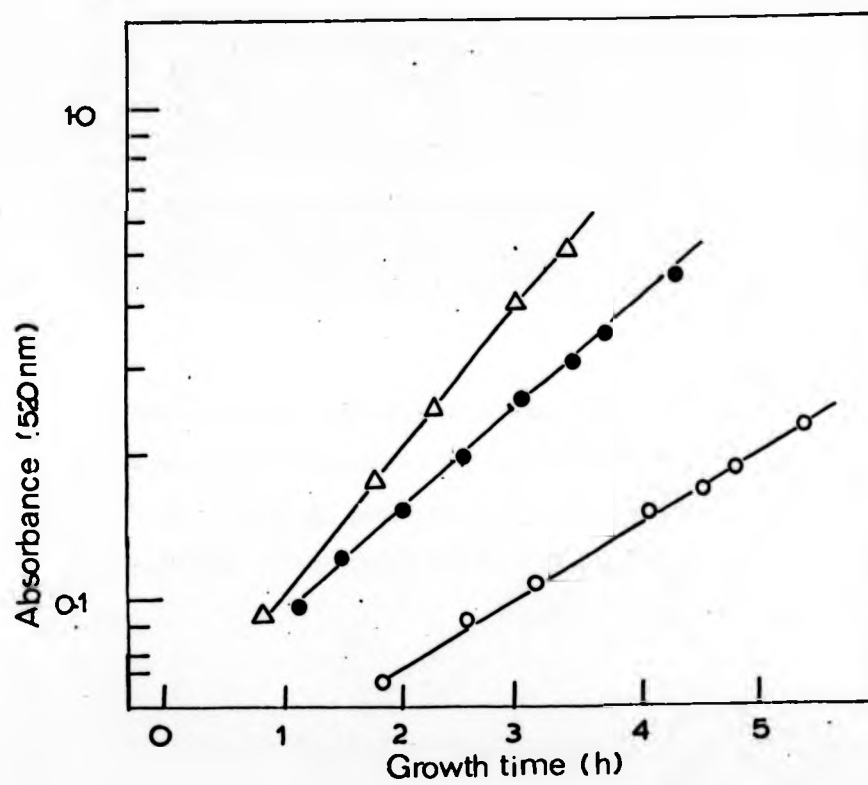
Strains had either never been grown in continuous-flow culture (a) or were isolated from chemostat culture experiments at $D = 0.1 \text{ h}^{-1}$ under carbon-limited (b) or phosphorous-limited conditions (c)

<u><i>E. coli</i> K12 with plasmid (resistance markers)</u>	μ_{max} (h^{-1})	Percentage increase of evolved strain μ_{max} compared to <u><i>E. coli</i> K12(TP120)</u>
TP120 ^a (Ap, Sm, Su, Tc)	0.4	-
TP120A ^b (Ap, Sm, Su)	0.56	40
TP120B ^c (Sm, Su, Tc)	0.46	5
TP120C ^c (Ap, Sm, Su)	0.57	43
TP120D ^c (Sm, Su)	0.47	18
TP120E ^c (no plasmid)	0.69	73
IR713 ^a (no plasmid)	0.64	60

Figure 3.8.1. Maximum specific growth rate of E.Coli K12 strains in closed culture in Davis and Mingioli defined medium. E. coli K12(TP120, (○), E. coli K12(TP120A), (●), and E. coli K12(TP120E) (no plasmid), (△).

Figure 3.8.1. Maximum specific growth rate of E.Coli K12 strains in closed culture in Davis and Mingioli defined medium. E. coli K12(TP120, (○), E. coli K12(TP120A), (●), and E. coli K12(TP120E) (no plasmid), (△).

(1)

E.Coli

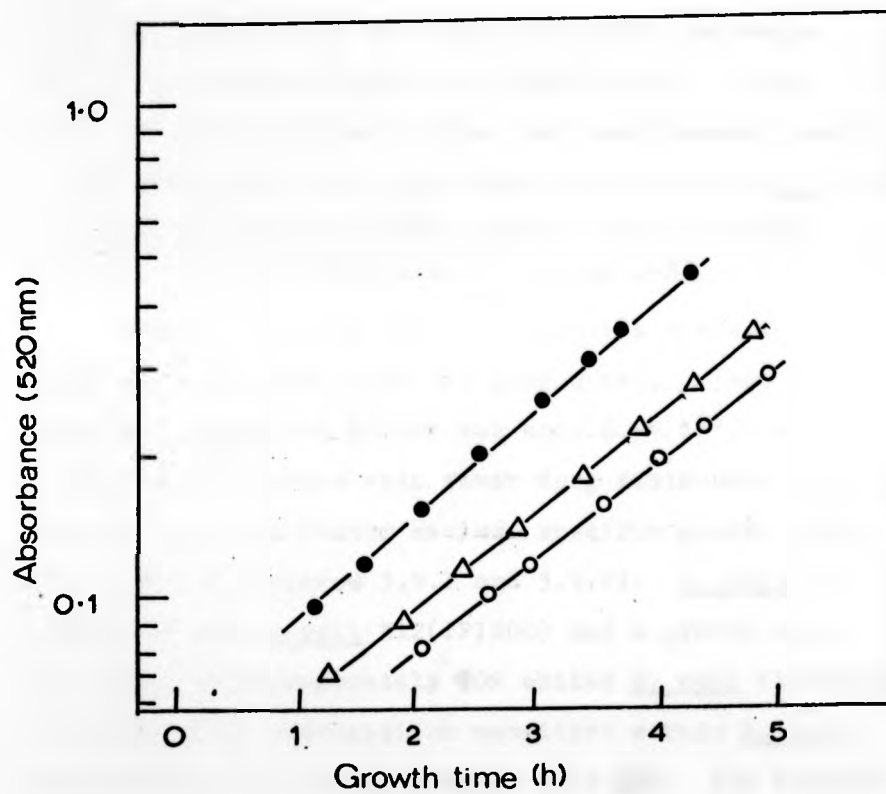
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Figure 3.8.2. Maximum specific growth rate of E. coli K12 strains in closed culture in Davis and Mingioli defined medium. E. coli K12(TP120B), (○); E. coli K12(TP120C), (●); and E. coli K12(TP120D), (△).

(2)



parent strain's μ_{\max} value. In contrast to the nutrient broth results E. coli K12(TP120B) and E. coli K12(TP120D) had growth advantages of only 15 to 18% compared to the 20% growth rate advantage in a rich medium (Tables 3.7. and 3.8.2). The evolved plasmid-minus strain had the greatest growth rate advantage in this defined medium with a μ_{\max} value of 0.69 h^{-1} which was 3% greater than the parent strain E. coli K12(TP120) $\mu_{\max} = 0.40 \text{ h}^{-1}$.

Table 3.8 shows that in Hershey's defined medium the maximum specific growth rate of the parent strain E. coli K12(TP120) was only 0.39 h^{-1} . All the evolved strains with fewer drug resistance markers were capable of faster maximum specific growth rates (Tables 3.8. Figures 3.9.1 and 3.9.2). E. coli K12(TP120B) and E. coli K12(TP120D) had a growth rate increase of approximately 40% whilst E. coli K12(TP120A) and the other tetracycline sensitive strain E. coli K12(TP120C) had an increase of only 23%. The plasmid-minus strain E. coli K12(TP120E) had a μ_{\max} value which was greater than any of the plasmid-carrying strains and was capable of growing at twice the rate of E. coli K12(TP120) (Table 3.8. Figures 3.9 and 3.9.2).

It could be considered that the selection and

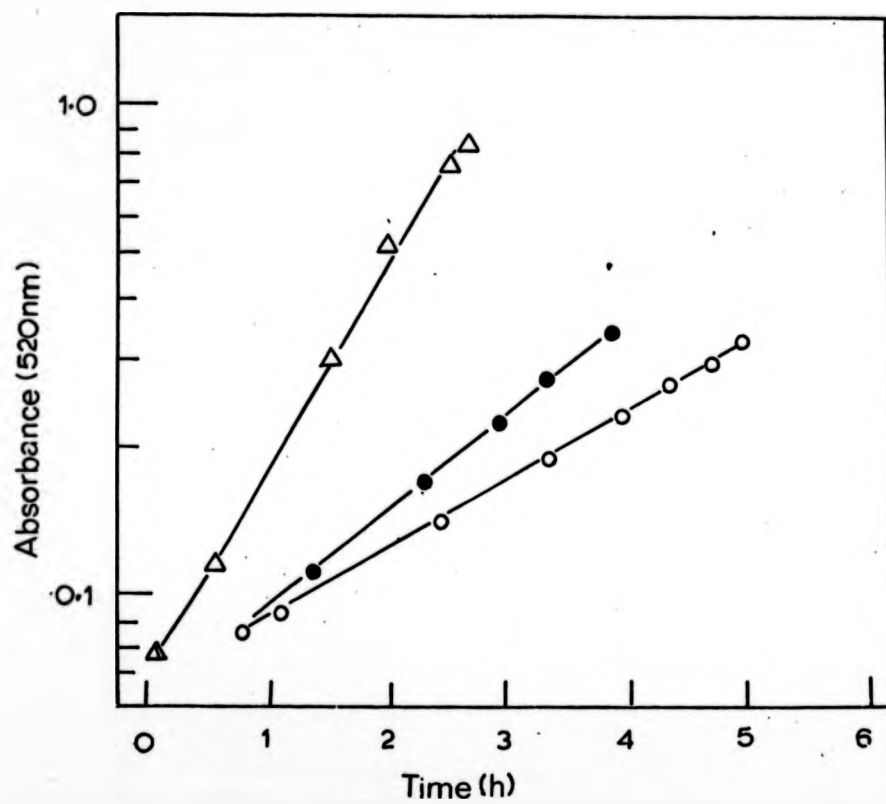
Table 3.8. Maximum specific growth rates for selected E. coli K12 strains carrying various drug resistance markers, or ncne, in closed culture in Hershey defined medium.

Strains had either not been grown in continuous-flow culture (a) or had been isolated from chemostat culture $D = 0.10 \text{ h}^{-1}$ under carbon-limited (b) or phosphorous-limited conditions (c)

<u>E. coli</u> K12 with plasmid (resistance markers)	μ_{max} (h^{-1})	Percentage increase of evolved strain μ_{max} compared to <u>E. coli</u> K12(TP120)
TP120 ^a (Ap, Sm, Su, Tc)	0.39	-
TP120A ^b (Ap, Sm, Su)	0.50	28
TP120B ^c (Sm, Su, Tc)	0.60	54
TP120C ^c (Ap, Sm, Su)	0.48	23
TP120D ^c (Sm, Su)	0.56	44
TP120E ^c (no plasmid)	0.90	131
IR713 ^a (no plasmid)	0.64	64

Figure 3.9.1. Maximum specific growth rate of E. coli K12 strains in closed culture in Hershey's defined medium. E. coli K12(TP120), (○); E. coli K12(TP120A), (●); and E. coli K12(TP120E) (no plasmid), (△).

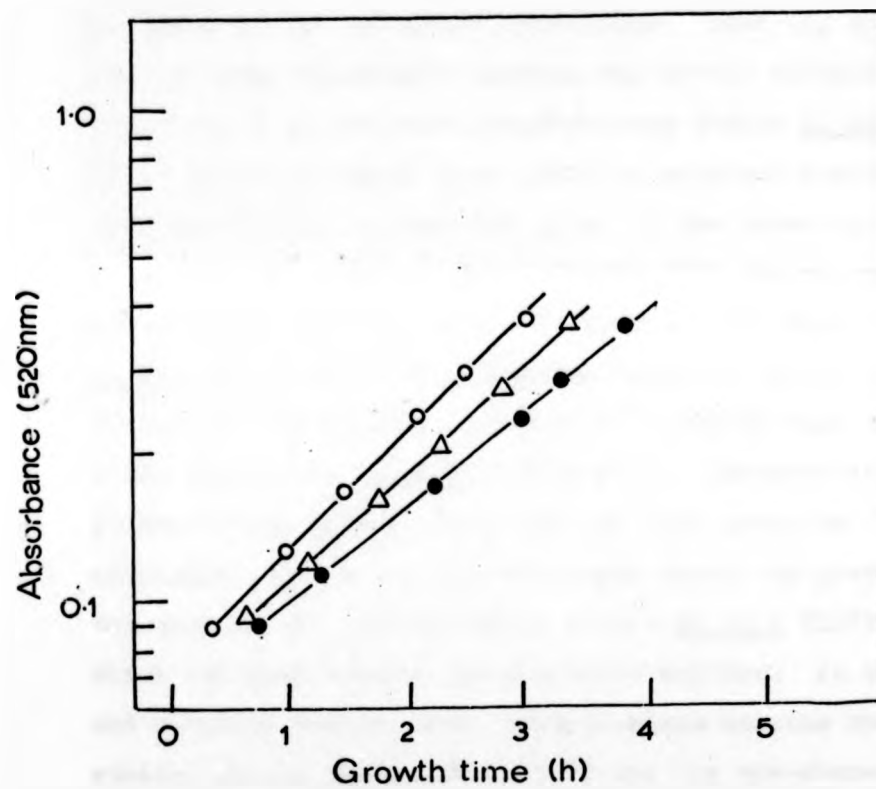
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Figure 3.9.2 Maximum specific growth rate of
E. coli K12 strains in closed culture in Hershey's
defined medium. E. coli K12(TP120B), (○);
E. coli K12 (TP120C), (●); E. coli K12(TP120D),
(△).

(2)



success of E. coli K12 strains which had lost antibiotic markers was due to the indirect result of selecting mutants capable of more efficient growth provided by the selection conditions. That is, the loss of drug resistance markers was merely coincidental. Accordingly an isogenic plasmid-minus strain E. coli K12 IR713 which has never been grown in nutrient-limited continuous-flow culture was grown in the three types of media. In nutrient broth (Table 3.6) the plasmid-minus strain did not appear to have an advantage since E. coli K12 IR713 had a maximum specific growth rate of 0.54 h^{-1} which was only 0.01 h^{-1} greater than the plasmid-carrying E. coli K12(TP120). Moreover the plasmid-minus strain which had not been grown in chemostat culture had a growth rate which was practically the same as the plasmid-minus strain E. coli K12(TP120E), which had been evolved in chemostat culture. In Davis and Mingioli medium both plasmid-minus strains had similar growth rates (Table 3.7) and the non-chemostat strain, E. coli K12 IR713, grew at a rate which was 60% faster than the same strain containing plasmid TP120. Furthermore, as expected from the growth rate of E. coli K12(TP120E) (no plasmid), the non-chemostat evolved strain also grew more rapidly than the other evolved strains resistant to two or three drugs (Table 3.7).

In Hershey's defined medium, whilst the non-chemostat evolved strain grew faster than the parent strain and any of the other strains (Table 3.8) its growth rate was considerably lower than the μ_{\max} for E. coli K12(TP120E) (no plasmid)

3.6. DISCUSSION

The purpose of the work described in this section was to examine the behaviour of the plasmid TP120 in a population of E. coli K12 in growth conditions where the presence of the resistance mechanisms was unnecessary and even an encumbrance. In drug-free medium the drug resistance functions coded for by the plasmid are redundant and environmental pressures could discriminate against the plasmid-carrying organisms in favour of plasmid-minus strains. However, the widespread nature of plasmids (Fredricq, 1963) and the diversity of functions coded for by plasmids suggest that plasmids could confer other unknown advantages on their bacterial hosts. These undetermined properties, that is, other than the drug resistance functions, might be of advantage to the bacteria in the drug-free nutrient-limited chemostat and could provide an explanation for the

very low frequency of appearance of plasmid-minus strains shown in this study. It is known that apart from the drug-resistance genes, the plasmid TP120 also codes for a UV resistance mechanism which is related to a decreased mutation rate and is obviously of survival value (Mortelmans and Stocker, 1976). The selection pressure therefore due to nutrient-limitation would be toward a smaller plasmid which would require less of the organism's elemental and energy resources required for maintenance of the plasmid and its associated RNA and protein synthesis. These resources could instead be diverted or increased to biomass production and a higher population growth rate. This principle was demonstrated by Zamenhof and Eichorn (1967) who showed that amino acid auxotrophs of Bacillus subtilis had a marked growth rate advantage over isogenic prototrophic strains under carbon-limited conditions. Furthermore the greater the number of biosynthetic sequences dispensed with by the auxotroph the greater was its growth rate advantage over the prototroph. Conversely organisms which wasted growth nutrients, for example by over production and excretion of amino acids were at a competitive disadvantage (Zamenhof and Eichorn, 1967; Baich and Johnson, 1968).

In view of the nutrient limited sub-maximal growth rate conditions imposed by chemostat continuous-flow culture and the large difference between specific growth rates of plasmid-carrying and plasmid-minus strains of E. coli K12 (Section 3.4) it was expected that mutant plasmid-minus strains would be rapidly selected for since they should rapidly outgrow the plasmid-carrying organisms. However the selection experiments demonstrated that this was not the case and that only in one instance under phosphorous-limited conditions after approximately 700 h growth in drug-free medium was a plasmid-minus strain detected. Similar observations of plasmid stability have been reported for chemostat culture (Melling et al., 1977; Wouters et al., 1978; Wouters and van Andel, 1979; Jones and Primrose, 1979; Adams et al., 1979) and in repeated batch subculture experiments (Falkow et al., 1977.; Dale and Smith, 1979). However, compared to these observations the plasmid TP120 was only partially stable since, particularly under phosphate-limited conditions, cultures readily lost resistance to one or more drugs which suggested that the plasmid could partially fragment perhaps producing a smaller plasmid coding only for an unknown, essential

advantageous function or alternatively an insertion occurred causing gene inactivation with a subsequent growth advantage over the parent strain. The discovery of transposons (Section 1.6) as DNA segments consisting of a replication origin and coding also for antibiotic resistance and insertion functions which can consequently insert into several sites on a genome have highlighted the recombination processes possible in plasmid-carrying organisms. (Cohen 1976). These transposable elements are not only capable of insertion into the genome but also excision and translocation to another site. The rate of loss of the tetracycline marker in batch culture in this study was very similar to the rate quoted for the tetracycline transposons and their possible role in the production of smaller plasmids of TP120 as found in nutrient-limited selection is discussed in a later section (Section 7.5).

Chemostat cultures were very complex with different populations carry plasmids with varying numbers of drug resistance markers being isolated from a single experiment. The distribution of these resistance markers between different populations, which were capable of more efficient growth than the parent strain E. coli K12(TP120) could account for the unusual washout

kinetics demonstrated for the disappearance of individual resistance markers from the population. This is because one was not simply observing the washout of a single non-competitive drug resistant population but instead the loss of drug resistance markers from a proportion of the population but a maintenance of the marker in another proportion of organisms in the total population. This situation would be further complicated if the plasmid TP120 and the evolved plasmids were transferred and retransferred within the chemostat mixed populations. Cullum et al. (1978a) has shown retransfer from recipient cells within separate closed-culture populations of E. coli carrying R100 F' lac plasmids. However these results indicate that this may have been an important factor in the selection experiments. Strongest evidence was provided by the observation that plasmid transfer was undetectable in liquid matings. This was characteristic of N group plasmids, with a value of 1×10^{-5} transconjugants per donor cells reported for a liquid mating in a study by Dennison and Baumberg (1975) involving plasmid-carrying E. coli. This value was raised to a frequency of 1×10^{-3} for a prolonged surface mating (Dennison and Baumberg, 1975; Burman, 1977). The chemostat culture was also stirred and aerated at

a very fast rate (Section 2.7) which would make the formation of mating pairs even more difficult.

Inefficient transfer in this plasmid group is due not to a slow initiation rate of transfer synthesis but to a difficulty in mating pair formation (Dennison and Baumberg, 1975). Only one tentative report has been made of the presence of N group specific pili on the surfaces of plasmid carrying organisms (Bradley, 1979). Other examinations of organisms carrying N group plasmids have failed to reveal the presence of N group specific pili and suggest that the N specific phage I_{Ke} appeared to attach directly to the cell surface (Brodt et al., 1974). Fractionated envelopes of an N group plasmid carrying E. coli K12 strain indicated the presence of an extra 3.1×10^4 dalton protein specific to plasmid gene transfer (Iyer, 1973). He further reported that plasmid TP120 increased the density of the outer membrane of an E. coli B/r strain and altered several inner and outer membrane proteins. This lack of defined sex pili means that donor and recipient cells have to be in very close proximity during conjugation which is more easily achieved on a solid medium. Alternatively in a recent study ~~by~~ Bradley (1979) ~~he~~ suggested that the difficulties encountered in isolation of N group pili is due to their fragile

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structure and this property would also account for their reduced transfer rate in liquid matings.

It has been suggested (Dennison and Baumberg, 1975) that increased surface mating efficiency was related to the natural habitat of N group plasmids, which are most commonly found in soil organisms. Falkow (1964) reported ~~that~~ improved transfer of the T⁺lac plasmid between Proteus mirabilis stains on solid media. Since N group plasmids constitute the major plasmid class found in Proteus-Providencia isolates, (Hedges, 1974), their fertility facilities may have been adapted to this ecological niche.

It is difficult to account for the reduced transfer rates for the evolved strains E. coli K12(TP120A) and E. coli K12(TP120B) reported in this study. It might be possible that growth in nutrient-limited continuous-flow culture had modified host cell membranes reducing further the efficiency of mating. It would be an interesting future study to determine if this corresponded to a reduction in infectivity of the N group specific filamentous phage I_{Ke} and was associated with changes in the membrane composition.

Decreased frequency of plasmid transfer in

defined medium compared to ^{with} nutrient broth also indicated that transfer was an unlikely process in continuous-flow culture selection experiments. Curtiss et al. (1969) confirmed that for F group plasmids in E. coli K12 host strains, growth in defined medium adversely influenced pili formation and the probability of pair formation. In a complex medium the organism had a greater proportion of its energy resources available for plasmid coded functions compared to growth in a ^{with} selectively defined medium which involved the burden of extra enzyme synthesis for initiation of cell growth processes.

The probability of plasmid spread by retransfer in continuous-flow culture is further reduced by the observation that nearly all the organisms isolated from the chemostat culture carried a plasmid, even if this was reduced in resistance markers. Cullum et al. (1978b) observed that the low mating efficiency of recipient cells which already carried an established plasmid was the main constraint on plasmid retransfer at low growth rates.

The loss of the original plasmid TP120 from the chemostat population appeared to be due to faster growth by the organisms carrying plasmids with fewer markers causing washout of the slower growing parent

population. It is difficult to explain this increase of growth efficiency simply in terms of conservation of limiting growth resources.

Preliminary experiments indicate that the growth advantage was not due to a more efficient nutrient assimilation system in evolved strains (Section 6).

The increased growth rate values for the mutant strains did not result, as predicted by kinetic theory, in

the total elimination of the uncompetitive parent strain. The maintenance of the original population

at low levels for extended periods had also been

reported in similar mixed populations experiments

with plasmid carrying strains (Melling *et al.*, 1977;

Wouters *et al.*, 1978; Wouters and van Anael, 1979)

although as a percentage of the population the

residual populations of E. coli K12(TP120) were several

orders of magnitude lower. For reasons outlined

earlier in this discussion it is unlikely that this

was caused by transfer within the population. Further-

more a residual resistant population was observed in a

study which involved a non-conjugative plasmid (Dale

and Smith, 1979). Since the plasmid TP120 usually

occurs in a single copy per cell,

it cannot save energy or elemental sources by a

reduction in copy number, but this may be an important

mechanism in those plasmids which exist in multiple copies within a cell and they could save energy not only by losing resistance markers but also by reducing the number of plasmid copies per cell.

It has been suggested that the original organism E. coli K12(TP120) persisted because of growth system artifacts, so that it was maintained, for example, as wall growth in culture vessels. However, when the surface growing population was examined for antibiotic resistance it was found to contain the same percentage of resistant organisms as in the liquid culture. It was possible that this parent strain maintained in the chemostat had undergone a mutation which favoured its attachment to the culture vessel wall enabling the parent population to survive in competition with the strains bearing fewer drug resistance markers. However the results summarised in Table 3.4 indicated that this was not the case. Alternatively it cannot be excluded that some population dependent mechanism so far unidentified exists to retain a low level of a potentially advantageous capability (drug resistance) within a population although the population is

temporarily disadvantaged. Clearly the long term survival of a population subjected to future drug challenges would be greatly increased if a few members of the population retained the necessary resistance mechanism.

4. TWO MEMBERED MIXED CULTURE COMPETITION EXPERIMENTS IN CONTINUOUS-FLOW CULTURE.

Closed culture experiments (Section 3.3) demonstrated that the five evolved strains, including the plasmid-minus strain, were capable of higher maximum specific growth rates than the parent strain E. coli K12(TP120). However these differences may not have been significant under substrate-limited conditions at submaximal growth rates although the fact that they were initially isolated in competition with E. coli (TP120) suggested that there was a relationship. Therefore the effect of both carbon and phosphorus as nutrient limitation and different imposed specific growth rates (dilution rates) on the outcome of competition for a single substrate between the parent E. coli K12(TP120) and the evolved strains separately in continuous-flow culture was investigated.

4.1. THE EFFECT OF INOCULATION PROCEDURE ON OUTCOME OF COMPETITION EXPERIMENTS IN CONTINUOUS-FLOW CULTURE.

Competition experiments in chemostat culture require that the newly inoculated populations were growing exponentially before establishment of continuous-flow conditions. This ensured that the differences in the growth dynamics of the competing populations were ascribable to

differences in growth parameters and not obscured by additional factors such as variable lag phases or differences in the physiological state of the population.

Two inoculation procedures (Section 2.11.) were adopted for comparison and demonstrated that the inoculation procedure affected the outcome of competition. One of the inoculation procedures involved the introduction of a batch grown culture of the evolved strain E. coli K12(TP120A) into an established steady state culture of the parent strain E. coli K12(TP120). In the second procedure the parent strain E. coli K12(TP120) and the evolved strain E. coli K12(TP120A) were grown separately in batch culture and then inoculated simultaneously into the chemostat culture vessel. A period of closed culture growth was maintained to ensure the populations were growing exponentially before medium flow was initiated.

The result of inoculating the evolved strain E. coli K12(TP120A) (Ap, Sm, Su,) into a steady state carbon-limited culture of the parent strain was that the established strain E. coli K12(TP120) (Ap, Sm, Su, Tc,) washed out of the chemostat very rapidly at a rate of -0.31 h^{-1} ($\mu_1 = -0.21 \text{ h}^{-1}$) compared to the washout rate of 0.13 h^{-1} ($\mu_1 = 0.03 \text{ h}^{-1}$) (Figure 4.2) in the experiment when both strains were inoculated simultaneously. (Table 4.1.). The loss of the parent organism E. coli K12 (TP120) was

Table 4.1. The effect of inoculation procedure and starting ratio on competition.

(a) E. coli K12(TP120) and E. coli K12(TP120A) inoculated simultaneously into the chemostat or (b) E. coli K12(TP120A) inoculated into a steady state culture of E. coli K12(TP120)

Dilution rate (h^{-1})	Nutrient limitation	Starting ratio (TP120): (TP120A)	Inoculation procedure	Rate of loss of uncompetitive population <u>E. coli</u> K12 (TP120) (h^{-1})	Growth rate of uncom- petitive population <u>E. coli</u> K12 (TP120) (h^{-1})	End Ratio (TP120): (TP120A)
0.15	phosphorus	0.83 : 1	a	-0.117	0.03	$2.6 \times 10^{-4} : 1$
0.15	phosphorus	1.02 : 1	a	-0.11	0.04	$8.7 \times 10^{-4} : 1$
0.25	phosphorus	0.73 : 1	a	-0.09	0.16	$8.6 \times 10^{-4} : 1$
0.25	phosphorus	0.016 : 1	a	-0.10	0.15	$3.3 \times 10^{-5} : 1$
0.1	carbon	1.46 : 1	a	-0.13	-0.03 (o)	$2.6 \times 10^{-4} : 1$
0.1	carbon	1.14 : 1	b	-0.314	-0.21 (o)	$8.2 \times 10^{-7} : 1$

measured by a fall in the number of colonies assayed on tetracycline containing plates (Figure 4.2.). The more rapid loss of the parent strain E. coli K12(TP120) observed as a result of using the first inoculation schedule was apparently not due to the proportion of the two competing strains at the onset of continuous-flow since in both experiments the ratio of the parent to evolved strains was approximately 1 : 1.(Table 4.1.).

Neither washout rate of the uncompetitive population nor the final ratio of competitive to uncompetitive populations achieved as result of washout seemed to be influenced by the original starting ratios of the two populations (Table 4.1.). At the dilution rate of 0.25 h^{-1} in phosphorus-limited conditions the uncompetitive population E. coli K12(TP120) washed out of chemostat culture at a rate of -0.10 h^{-1} (equivalent to a specific growth rate of 0.15 h^{-1}). The same rate of washout was observed when the competitive and uncompetitive populations were equal in number or if E. coli K12(TP120) was outnumbered 10 : 1 by the successful tetracycline sensitive organism E. coli K12 (TP120A). A further interesting observation was that in almost all of the continuous-flow culture competition experiments, there was a period of growth, up to 50 h after initiation of medium flow, when the ratio of the two populations fluctuated (Figures 4.1, 4.7 and 4.8). before the uncompetitive population was washed out of the

Figure 4.1. Competition between E. coli K12(TP120) and E. coli K12(TP120A) at $D = 0.35 \text{ h}^{-1}$ in carbon-limited chemostat culture. Total number of organisms (○) and number of tetracycline resistant organisms (E. coli K12(TP120)), (●)

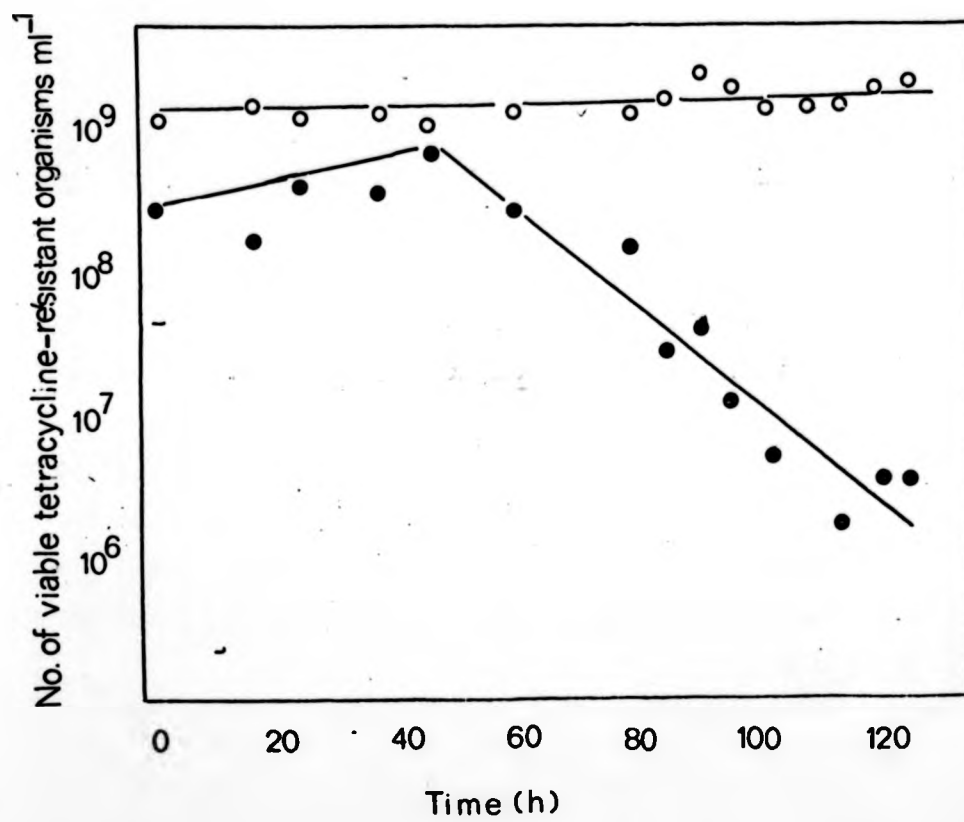
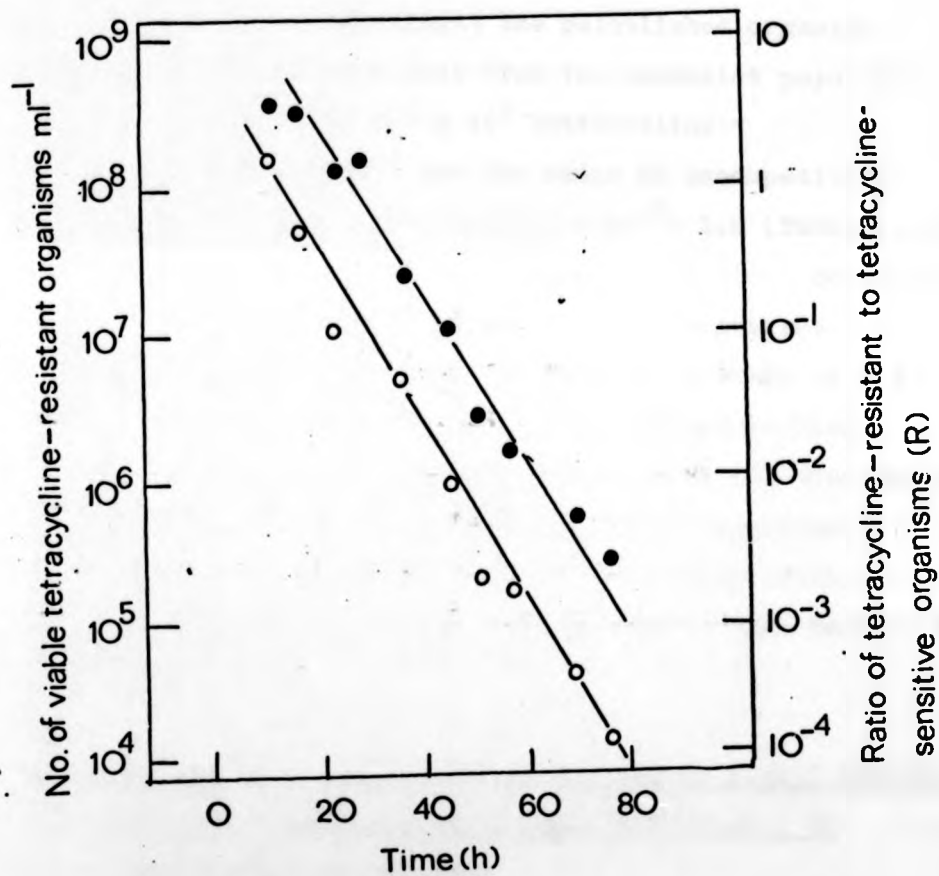


Figure 4.2. Competition between E. coli K12(TP120) and E. coli K12(TP120A) at $D = 0.10h^{-1}$ in carbon-limited chemostat culture. Number of viable tetracycline-resistant organisms (E. coli K12(TP120)) (o) and ratio of the number of tetracycline resistant organisms (E. coli K12(TP120)) to tetracycline sensitive organisms (E. coli K12(TP120A)) (●).



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chemostat at a constant rate. In all cases described in this section the washout rate was calculated from the slope which had been fitted by least squares regression.

In the first experiment the established organism E. coli K12(TP120) was lost from the chemostat population until there were only 8.8×10^2 tetracycline resistant bacteria ml^{-1} and the ratio of uncompetitive to competitive population was $8.2 \times 10^{-7} : 1.0$ (Table 4.1.). In the simultaneously inoculated culture a final concentration of 1×10^4 tetracycline resistant parent organisms E. coli K12(TP120) was achieved which is a ratio of $1 \times 10^{-5} : 1$ for uncompetitive to competitive populations (Figure 4.2). As in continuous-flow selection experiments (Section 3.1.3.2) and in later competition experiments (Section 4.2., 4.3., and 4.4) this small population of the uncompetitive organisms was maintained until termination of the experiment.

4.2. COMPETITION BETWEEN THE PARENT STRAIN E. COLI K12(TP120) AND THE EVOLVED STRAIN E. COLI K12(TP120A) IN CONTINUOUS-FLOW CULTURE

A detailed examination was made of the kinetics of competition between the parent strain E. coli K12(TP120) and the tetracycline sensitive strain E. coli K12(TP120A) which had been isolated from a carbon -limited selection

experiment. Phosphate and carbon-limitation conditions were examined in a number of experiments at dilution rates between 0.10 h^{-1} to 0.35 h^{-1} .

4.2.1. Carbon-limited chemostat culture.

Table 4.2 shows that in carbon-limited conditions at all the specific growth rates (dilution rates) examined, the tetracycline sensitive population E. coli K12(TP120A) always had a competitive growth advantage over the resistant parent population E. coli K12(TP120). Loss of the parent population E. coli K12(TP120) (Ap, Sm, Su, Tc) was measured by the decline in viable count for organisms able to grow on tetracycline containing agar plates. Figures 4.1., 4.2 and 4.3 show that as in the chemostat selection experiments, the noncompetitive parent population was never completely removed from the chemostat culture. Instead the number of tetracycline resistant organisms declined at a constant rate over a period of 80 h until a level of approximately 1×10^4 organisms ml^{-1} was reached. At this stage the parent strain constituted only 0.01% of the total chemostat populations and was maintained at levels varying between .01 to 0.1% of the total population until the termination of the experiment.

The degree of competitiveness of the evolved organism E. coli K12(TP120A) increased with a decreasing dilution

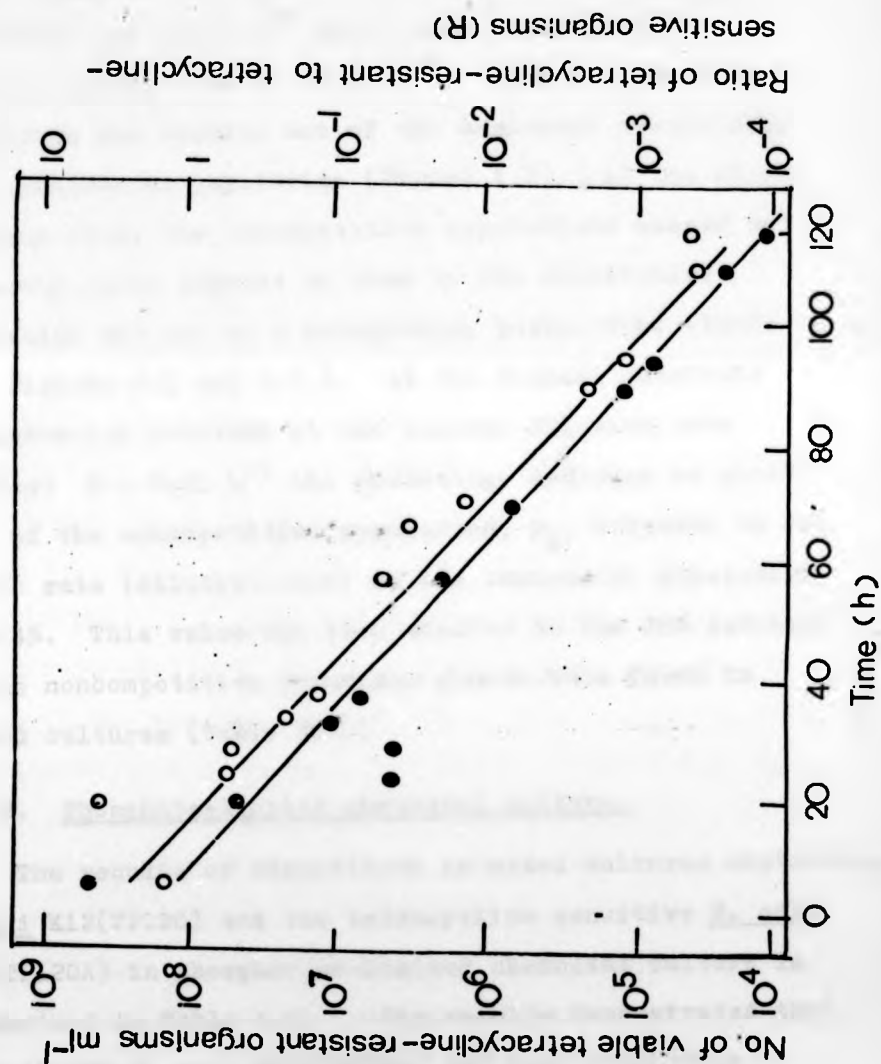
Table 4.2 Competition experiments between E. coli K12(TP120) and E. coli (TP120A) at different dilution rates in carbon-limited and phosphorous-limited chemostat cultures.

Nutrient limitation	Dilution Rate (h^{-1})	Rate of loss of uncompetitive population from slope of		Specific growth rate, μ , of uncompetitive population:		Average Percentage decrease of μ for uncompetitive population
		No. of TcR organisms (h^{-1})	Ratio $TcR : Tc^s$ (h^{-1})	<u>E. coli</u> K12 (TP120) Eqn 1 Jannasch(1969)	<u>E. coli</u> K12 (TP120) Eqn 3 Slater & Bull (1978)	
Carbon (glucose)	0.10	-0.12	-0.13	-0.02 (o)	-0.03 (o)	100
	0.15	-0.10	-0.10	0.05	0.05	67
	0.25	-0.08	-0.08	0.17	0.17	32
	0.35	-0.08	-0.08	0.27	0.26	24
Phosphor (phosphate)	0.10	-0.10	-0.09	0.01	0.02	85
	0.15	-0.12	-0.11	0.03	0.04	77
	0.25	-0.09	-0.09	0.16	0.16	36
	0.35	-0.12	-0.14	0.23	0.21	37

Figure 4.3. Competition between E. coli K12(TP120) and E. coli K12(TP120A) at $D = 0.25 \text{ h}^{-1}$ in carbon-limited chemostat culture. Number of tetracycline-resistant organisms (E. coli K12(TP120)), (o) and ratio of the number of tetracycline-resistant organisms (E. coli K12(TP120)) to tetracycline-sensitive organisms (E. coli K12(TP120A)). (●).



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rate (Table 4.2). At a dilution rate of 0.10 h^{-1} the rate of loss of the uncompetitive population E. coli K12(TP120) was -0.12 h^{-1} which gave a calculated specific growth rate of -0.02 h^{-1} . That is, the parent population was washing out of the chemostat essentially as a non-growing population (Figure 4.2). At the higher dilution rates the uncompetitive populations washed out at growth rates imposed on them by the competitive population but not as a non-growing population. (Table 4.2; Figures 4.1 and 4.3.). At the highest substrate concentration obtained at the highest dilution rate examined $D = 0.35 \text{ h}^{-1}$ the percentage decrease in growth rate of the uncompetitive population, μ_1 , compared to the growth rate (dilution rate) of the successful population was 24%. This value was very similar to the 28% decrease in the noncompetitive organisms growth rate found in closed cultures (Table 3.7.)

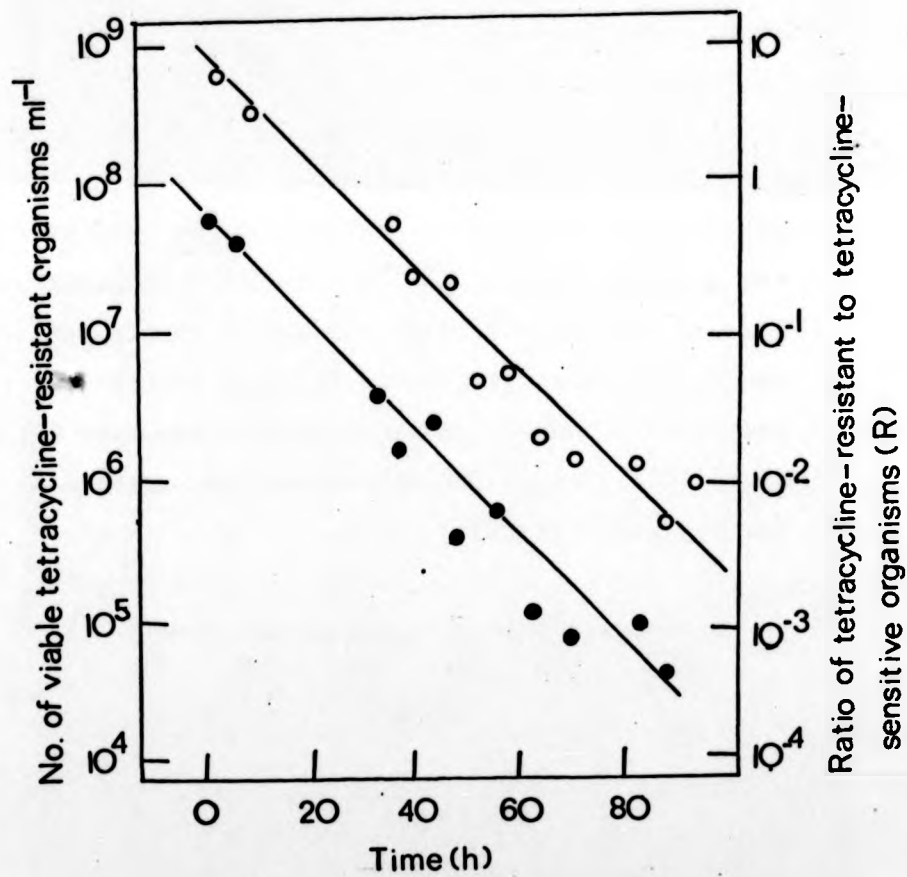
4.2.2. Phosphate-limited chemostat culture.

The results of competition in mixed cultures containing E.coli K12(TP120) and the tetracycline sensitive E. coli K12(TP120A) in phosphorus-limited chemostat culture is summarised in Table 4.2. The results demonstrated that even though E. coli K12(TP120A) had been originally isolated from a carbon-limited selection experiment this strain also had a growth advantage under phosphorus-limited conditions.

At high and low dilution rates the parent population E. coli K12(TP120) washed out from the chemostat culture at a constant rate. At a dilution rate of 0.10 h^{-1} (Figure 4.4) washout continued for 120 h at a rate of $- 0.09 \text{ h}^{-1}$ (equivalent to a specific growth rate of 0.01 h^{-1} for the uncompetitive population E. coli K12(TP120)), until a final count of 1×10^4 tetracycline resistant organisms ml^{-1} was reached. At $D = 0.25 \text{ h}^{-1}$ the uncompetitive organism was lost from the chemostat population at the same rate of $- 0.09 \text{ h}^{-1}$. (Figure 4.5) which continued over a period of approximately 80 h until the parent organism was outnumbered by a ratio of 100 : 1 by the evolved strain E. coli K12(TP120A).

Although the rate of washout was the same over the dilution rate range, $D = 0.10 \text{ h}^{-1}$ to 0.35 h^{-1} , the competitiveness of E. coli K12(TP120A) increased with decreasing dilution rate such that at $D = 0.10 \text{ h}^{-1}$ there was an 85% decrease in the specific growth rate value of E. coli K12(TP120) compared with that of E. coli K12(TP120A) (Table 4.2. and Figure 4.4). At the highest dilution rate of 0.35 h^{-1} the percentage decrease in the growth rate of the uncompetitive strains was 37% which was significantly higher than the 22% decrease in the μ_{max} value of E. coli K12(TP120) compared to E. coli K12(TP120A) recorded for closed culture growth in Hershey glucose salts medium.

'Figure 4.4 Competition between E. coli K12(TP120) and E. coli K12(TP120A) at $D = 0.10 \text{ h}^{-1}$ in phosphate-limited chemostat culture. Number of viable tetracycline-resistant organisms (E. coli K12(TP120)), (\circ) and ratio of number of tetracycline-resistant (E. coli K12(TP120)) to tetracycline sensitive organisms (E. coli K12(TP120A)) (\bullet).



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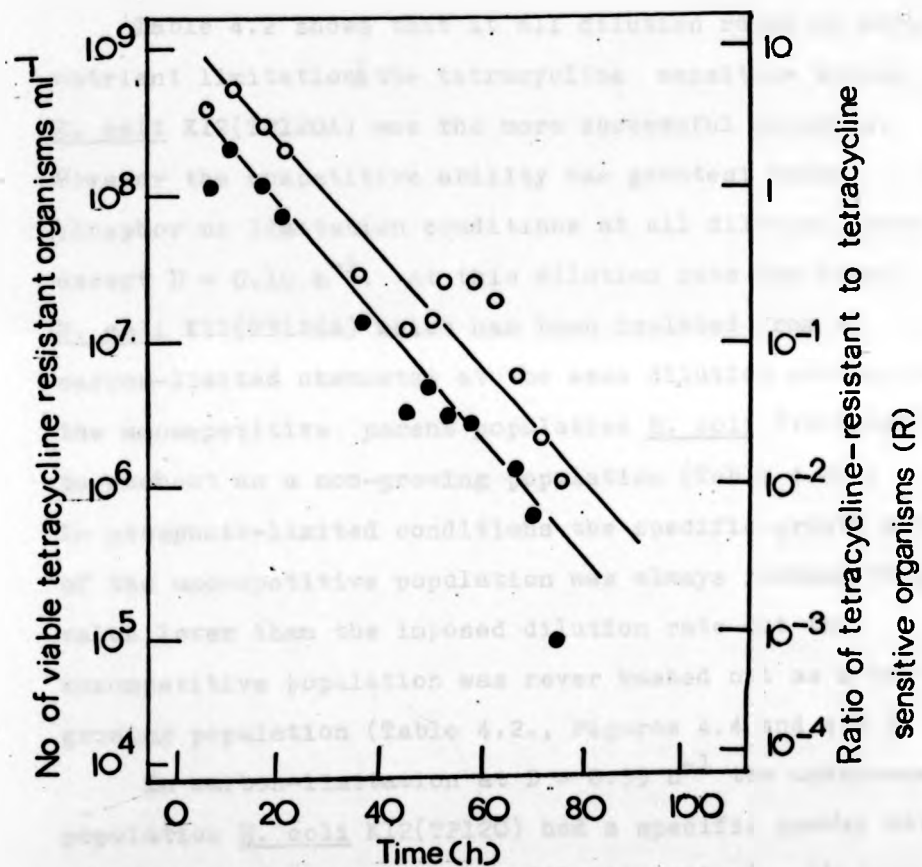
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Figure 4.5. Competition between E. coli K12(TP120) and E. coli K12(TP120A) at $D = 0.25 \text{ h}^{-1}$ in phosphate-limited chemostat culture. Number of viable tetracycline-resistant organisms (E. coli K12(TP120)) (o) and ratio of number of tetracycline-resistant (E. coli K12(TP120)) to tetracycline-sensitive organisms (E. coli K12(TP120A)) (●).



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4.2.3. A comparison of competition in carbon and phosphorus limited chemostat culture.

Table 4.2 shows that at all dilution rates in both nutrient limitations the tetracycline sensitive strain E. coli K12(TP120A) was the more successful organism. However the competitive ability was greatest under phosphorus limitation conditions at all dilution rates except $D = 0.10 \text{ h}^{-1}$. At this dilution rate the strain E. coli K12(TP120A) which has been isolated from a carbon-limited chemostat at the same dilution rate caused the uncompetitive parent population E. coli K12(TP120) to washout as a non-growing population (Table 4.2.). In phosphate-limited conditions the specific growth rate of the uncompetitive population was always reduced to a value lower than the imposed dilution rate but the uncompetitive population was never washed out as a non-growing population (Table 4.2., Figures 4.4 and 4.5.).

In carbon-limitation at $D = 0.35 \text{ h}^{-1}$ the unsuccessful population E. coli K12(TP120) had a specific growth rate which was 24% lower than the dilution rate (or the specific growth rate) of E. coli K12(TP120A). The growth differences in batch culture are of the same order with a 28% decrease in the μ_{max} value of E. coli K12(TP120) compared with the tetracycline sensitive E. coli K12(TP120A). In contrast in closed culture maximum specific growth rate values in

Hershey's defined medium with excess phosphate there was only a 22% decrease in the μ_{\max} value of the parent strain compared to the evolved strain E. coli K12(TP120A). Batch culture results therefore suggest that E. coli K12(TP120A) would be the most successful competitor in carbon-limited chemostat experiments. However at $D = 0.35 \text{ h}^{-1}$ in phosphate limited culture the growth rate of E. coli K12(TP120) was reduced to a value of 0.21 h^{-1} which was 37% lower than the growth rate of the successful organisms E. coli K12(TP120A) (Table 4.2).

4.3 COMPETITION BETWEEN THE PARENT STRAIN E. COLI K12(TP120) AND OTHER EVOLVED STRAINS IN CONTINUOUS-FLOW CULTURE UNDER PHOSPHORUS-LIMITED CONDITIONS.

Following the success of the carbon-limited evolved strain E. coli K13(TP120A) in competition with the parent strain E. coli K12(TP120) in carbon and phosphorus-limited chemostat cultures, the outcome of competition between the strains E. coli K12(TP120B), E. coli K12(TP120D) and E. coli K12(TP120E) (no plasmid) evolved under phosphate-limited conditions with the parent organism E. coli K12(TP120) was examined under phosphate-limited conditions.

In mixed populations containing the parent strain E. coli K12(TP120) (Ap, Sm, Su, To,) and E. coli K12(TP120B) (Sm, Su, To) the parent population was assayed on ampicillin-

containing agar plates while with E. coli K12(TP120D) (Sm, Su) and E. coli K12(TP120E) (no plasmid) plates containing tetracycline were used.

Under phosphorus-limited conditions at dilution rates ranging from 0.10 h^{-1} to 0.35 h^{-1} the evolved strains carrying fewer resistance markers were always the more successful competitive organisms against E. coli K12(TP120) (Tables 4.3, 4.4 and 4.5). The parent organism E. coli K12(TP120) behaved in a similar fashion to the competition experiments with E. coli K12(TP120A) and washed out of the chemostat at a constant rate until a level of approximately 1.0×10^2 to 1.0×10^3 parent organisms ml^{-1} was reached. These levels were maintained until the termination of the experiments (Figure 4.6)

In competition with E. coli K12(TP120B) (Sm, Su, Tc) the degree of the competitiveness of the successful population appeared to increase with substrate concentration, that is, with increasing dilution rate, such that at $D = 0.35 \text{ h}^{-1}$ the specific growth rate of the uncompetitive population (0.17 h^{-1}) was 51% lower than the dilution rate while at $D = 0.10 \text{ h}^{-1}$ the difference was only 41%. These differences were greater than the 35% difference in the μ_{max} values recorded for the two strains in closed culture when phosphate was supplied in excess.

Table 4.3 Competition experiments between E. coli K12(TP120) and E. coli K12(TP120B) at different dilution rates in phosphorous-limited chemostat culture.

Dilution rate (h^{-1})	Rate of loss of uncompetitive population $\frac{E. coli \text{ K12 (TP120)}}{(TP120)}$ (h^{-1})	Specific growth rate μ of uncompetitive <u>E. coli</u> K12 $\frac{(TP120)}{(TP120)}$ (h^{-1})	Maximum specific growth rate of competitive population (h^{-1})	Average % decreases of μ for uncompetitive population $\frac{E. coli \text{ K12 (TP120)}}{(TP120)}$
0.1	-0.041	0.059	0.60	41
0.15	-0.082	0.068	0.60	60
0.35	-0.180	0.170	0.60	51

Competition with E. coli K12(TP120D) (Sm, Su) and E. coli (TP120) was examined at $D = 0.10 \text{ h}^{-1}$ and $D = 0.35 \text{ h}^{-1}$ (Table 4.4.). At both dilution rates E. coli K12 (TP120D) was a much more competitive organism than suggested by the μ_{max} values determined in batch culture which gave greater rate differences of 30%. At a dilution rate of 0.10 h^{-1} the parent population washed out of the chemostat apparently as a non growing and, indeed, lysing population since the loss of this populations occurred with a slope of -0.24 h^{-1} . At $D = 0.35 \text{ h}^{-1}$ with higher phosphorus concentration E. coli (TP120) only managed growth with a specific growth rate of 0.01 h^{-1} .

The plasmid-minus strain E. coli K12 (TP120E) (no plasmid) which was originally isolated from a phosphate-limited chemostat culture had the greatest growth advantage compared to E. coli K12(TP120) when measured in batch culture (Table 3.9). In continuous - flow culture E. coli K12 (TP120E) (no plasmid) was again the most competitive organism (Table 4.5; Figure 4.6) with the exception of the surprising results produced in competition experiments involving E. coli K12(TP120) and E. coli K12(TP120D). E. coli K12(TP120E) (no plasmid) was most competitive at the lowest dilution rate with the parent organisms washing out of culture at

Table 4.4 Competition experiments between E. coli K12(TP120) and E. coli K12(TP120D) at different dilution rates in phosphor us-limited chemostat culture.

Dilution rate (h^{-1})	Rate of loss of uncompetitive population <u>E. coli</u> K12 (TP120) (h^{-1})	Specific growth rate μ of uncompetitive populations (h^{-1})	Maximum specific growth rate μ_{max} of competitive populations (h^{-1})	Average % decrease of μ for uncompetitive populations
0.10	-0.24	-0.14 (0)	0.56	100 %
0.35	-0.34	0.01	0.56	97 %

Table 4.5 Competition experiments between E. coli K12(TP120) and E. coli K12(TP120E) (no plasmid) at different dilution rates in phosphorus us-limited chemostat culture.

Dilution rate (h^{-1})	Rate of loss of uncompetitive population <u>E. coli</u> K12 (TP120) (h^{-1})	Specific growth rate μ of uncompetitive population (h^{-1})	Maximum specific growth rate μ_{max} of competitive population (h^{-1})	Average % decrease of μ for uncompetitive population
0.10	-0.175	-0.075 (o)	0.90	100
0.25	-0.12	0.13	0.90	48
0.35	-0.26	0.09	0.90	74

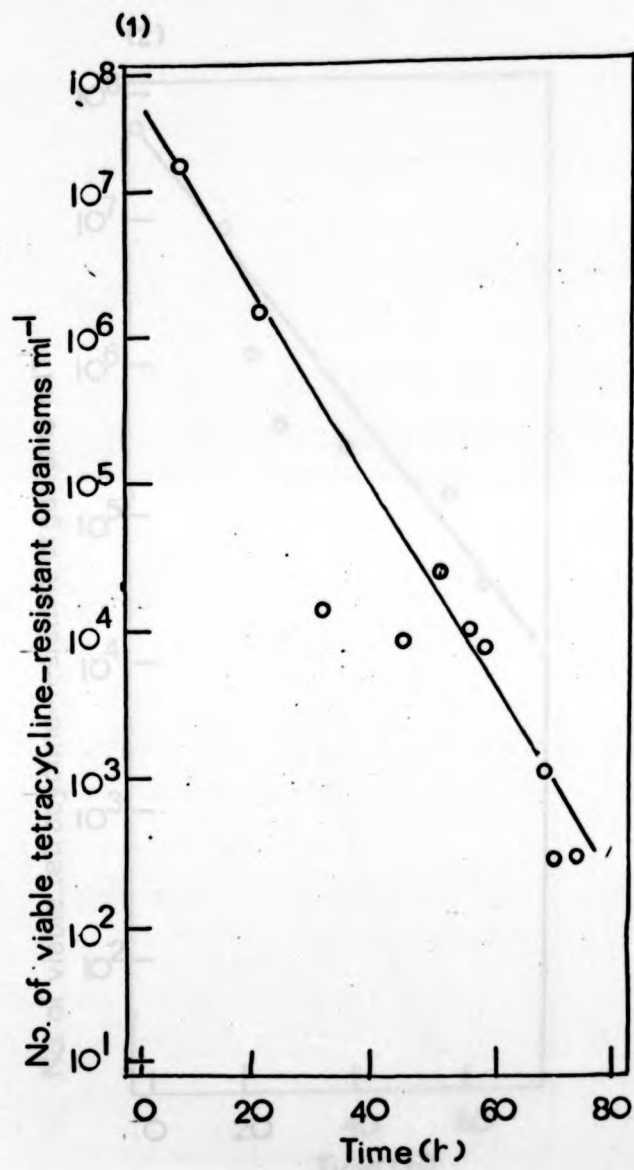
a rate of -0.175 h^{-1} which was probably equivalent to a non-growing population (Figure 4.6.1.). At $D = 0.35 \text{ h}^{-1}$ the parent organism E. coli K12(TP120) had a calculated growth rate of 0.09 h^{-1} as it was eliminated from the chemostat culture at a rate of -0.26 h^{-1} (Figure 4.6.2). The percentage decrease of the parent population growth rate compared to E. coli K12(TP120E) (no plasmid) was greater than the batch growth value of 56% at $D = 0.10 \text{ h}^{-1}$ and $D = 0.35 \text{ h}^{-1}$ but surprisingly at $D = 0.25 \text{ h}^{-1}$ it was slightly less at 48% (Table 4.5). At $D = 0.25 \text{ h}^{-1}$ the parent population decreased at a constant rate until a concentration of 1.0×10^4 organisms ml^{-1} was reached while at the remaining dilution rates the parent population reached a final level of approximately $1 \times 10^2 \text{ ml}^{-1}$.

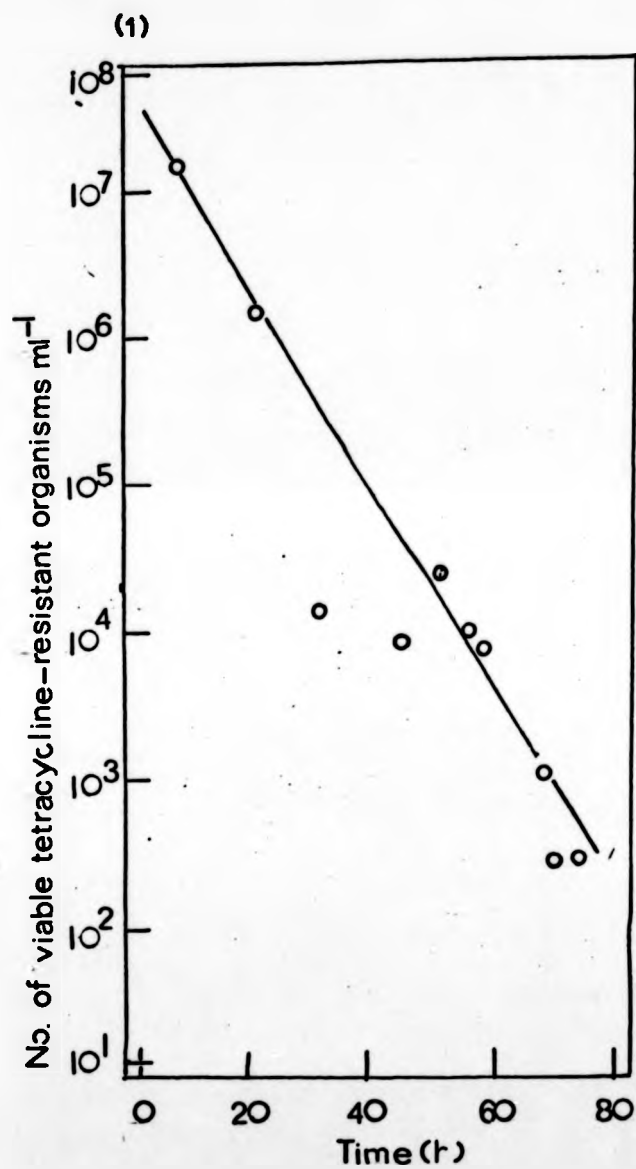
4.3.1. A comparison of the effect of carbon-limited strains and phosphate-limited evolved strains on competition.

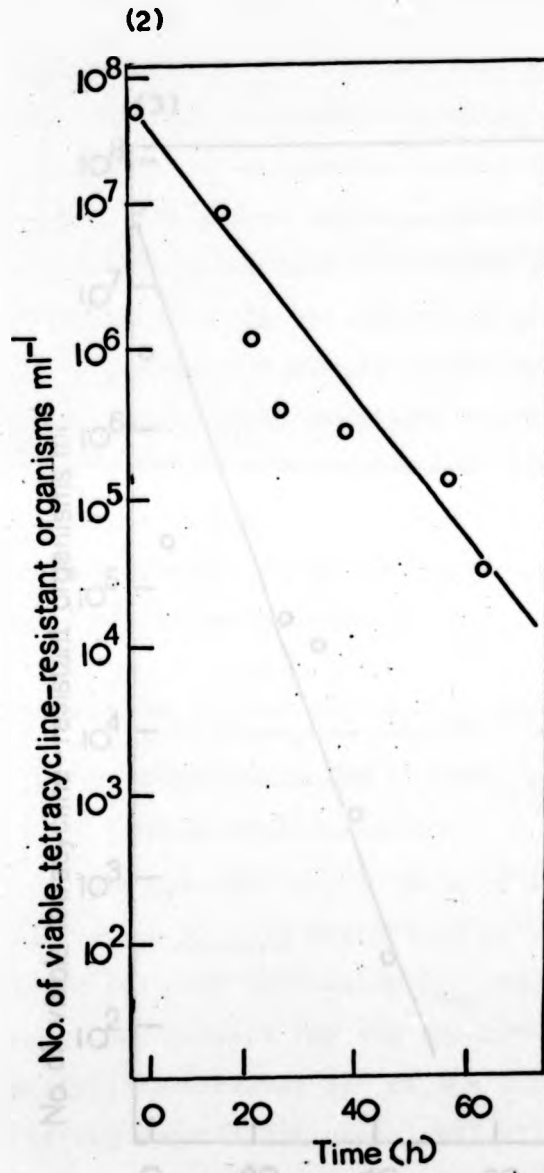
Results of competition experiments involving the parent strain E. coli K12(TP120) and strains evolved from carbon-limited and phosphorous-limited chemostat cultures have shown that under all conditions examined E. coli K12(TP120) was the unsuccessful organism.

The strain E. coli K12(TP120A) evolved under carbon-

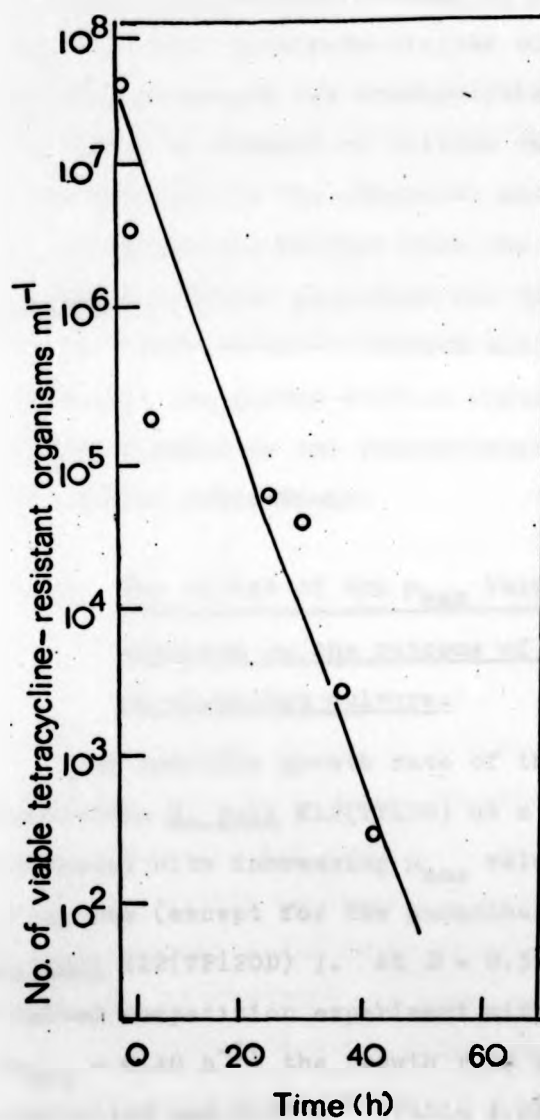
Figure 4.6 Competition between E. coli K12(TP120) and E. coli K12(TP120E) (no plasmid) in phosphate-limited chemostat culture at (1) $D = 0.10 \text{ h}^{-1}$; (2) $D = 0.20 \text{ h}^{-1}$; (3) $D = 0.35 \text{ h}^{-1}$. Number of viable tetracycline-resistant organisms (E. coli K12(TP120)) (o).







(3)



limited conditions (Section 4.2) was a successful competitor in carbon and phosphorus-limited chemostat experiments. Moreover under phosphate-limited conditions, E. coli K12(TP120A) caused the uncompetitive organism E. coli K12(TP120) to washout of culture immediately upon initiation of medium flow to the chemostat and at a faster rate than in carbon-limited culture when the unsuccessful organism E. coli K12(TP120) persisted for 50 h in chemostat culture before washout (Figure 4.1). Apart from this difference the carbon-evolved organism behaved in a similar fashion to the phosphate-evolved organisms under both growth limitations.

4.3.2. The effect of the μ_{\max} value of the competitive organism on the outcome of competition experiments in chemostat culture.

The specific growth rate of the uncompetitive population E. coli K12(TP120) at a given dilution rate decreased with increasing μ_{\max} values of the competitive organisms (except for the anomalous result described for E. coli K12(TP120D)). At $D = 0.35 \text{ h}^{-1}$ in a phosphate-limited competition experiment with E. coli K12(TP120A) ($\mu_{\max} = 0.48 \text{ h}^{-1}$) the growth rate (μ_1) of the uncompetitive population was 0.23 h^{-1} (Table 4.2). The value of μ_1 was reduced to 0.17 h^{-1} in competition with E. coli K12(TP120B)

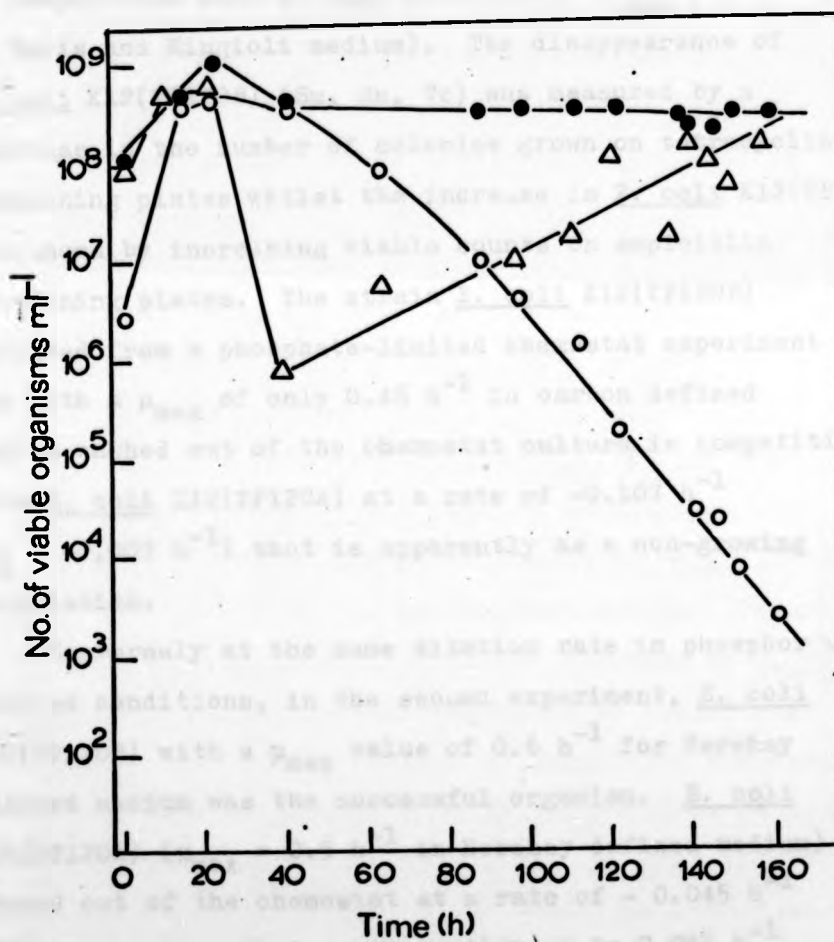
(μ_{\max} 0.60 h^{-1}) and with the faster growing plasmid-minus strain E. coli K12(TP120E) μ_1 was reduced to a value of only 0.09 h^{-1} (Table 4.5).

4.4. COMPETITION BETWEEN EVOLVED STRAINS IN PHOSPHATE AND CARBON-LIMITED CONDITIONS IN CONTINUOUS-FLOW CULTURE.

It was necessary to examine further the effect of the origin of the evolved strains (that is, whether from carbon or phosphate selection experiments) and their μ_{\max} values on the outcome of competition. Accordingly separate competition experiments were designed with the carbon-limited chemostat evolved strain E. coli K12(TP120A) and the phosphate-limited evolved strains E. coli K12(TP120B) and E. coli K12(TP120D). In experiment 1 E. coli K12(TP120A) and E. coli K12(TP120B) were grown in mixed culture at $D = 0.10 \text{ h}^{-1}$ in carbon-limited medium whilst in a second experiment the two strains were again grown together but this time in phosphate-limited conditions and the difference in outcome of competition recorded. In a third experiment the carbon limited selection experiment evolved strain E. coli K12(TP120A) was grown in competition with E. coli K12(TP120D) at the low dilution rate of $D = 0.10 \text{ h}^{-1}$ in carbon limited-medium.

Figure 4.7 shows the result of competition between E. coli K12(TP120A) and E. coli K12(TP120B) in carbon-limited conditions at $D = 0.10 \text{ h}^{-1}$. E. coli K12(TP120A)

Figure 4.7. Competition between E. coli K12(TP120A) and E. coli K12(TP120B) at $D = 0.10 \text{ h}^{-1}$ in carbon-limited chemostat culture. Total number of organisms (●); number of viable tetracycline resistant organisms (E. coli K12(TP120B)) (○) and number of viable ampicillin resistant organisms (E. coli K12(TP120A)) (Δ).



originally isolated from a carbon-limited selection experiment and with a μ_{\max} value of 0.56 h^{-1} in Davis and Mingioli medium was the successful organism in competition with E. coli K12(TP120B) ($\mu_{\max} = 0.46 \text{ h}^{-1}$ in Davis and Mingioli medium). The disappearance of E. coli K12(TP120B) (Sm, Su, Tc) was measured by a decrease in the number of colonies grown on tetracycline-containing plates whilst the increase in E. coli K12(TP120A) was shown by increasing viable counts on ampicillin containing plates. The strain E. coli K12(TP120B) isolated from a phosphate-limited chemostat experiment and with a μ_{\max} of only 0.46 h^{-1} in carbon defined medium washed out of the chemostat culture in competition with E. coli K12(TP120A) at a rate of -0.107 h^{-1} ($\mu_1 = -0.007 \text{ h}^{-1}$) that is apparently as a non-growing population.

Conversely at the same dilution rate in phosphorus limited conditions, in the second experiment, E. coli K12(TP120B) with a μ_{\max} value of 0.6 h^{-1} for Hershey defined medium was the successful organism. E. coli K12(TP120A) ($\mu_{\max} = 0.5 \text{ h}^{-1}$ in Hershey defined medium) washed out of the chemostat at a rate of -0.045 h^{-1} with a specific growth rate equivalent to 0.055 h^{-1} (Figure 4.8).

A feature of both nutrient limitations was the

apparent increase of the non-competitive population at a rate of approximately 0.04 h^{-1} before washing out from the mixed population (Figure 4.7 and 4.8). In the phosphate-limited chemostat (Figure 4.8) the ampicillin-resistant E. coli K12(TP120A) had not grown successfully during the closed culture period of growth prior to onset of medium flow such that at the initiation of flow there were only 4×10^4 ampicillin resistant organisms ml^{-1} in the culture. The organisms then increased to a concentration of 5×10^6 organisms ml^{-1} before finally washing out.

In experiment number 3, at the same dilution rate in carbon-limited conditions E. coli K12(TP120A) was again the successful organism in competition with the streptomycin and sulphonamide resistant E. coli K12(TP120D) ($\mu_{\text{max}} = 0.47 \text{ h}^{-1}$ in Davis and Mingioli medium) (Figure 4.9). Due to the lack of sufficient markers on E. coli K12(TP120D) (Sm, Su) it was impossible to follow its decline directly but the number of ampicillin resistant organisms, that is E. coli K12(TP120A) could be assayed on ampicillin containing agar plates. E. coli K12(TP120A) increased from 1×10^4 to 1×10^7 resistant organisms ml^{-1} at a rate of 0.066 h^{-1} to become the dominant population after 160 h of growth (Figure 4.9).

Figure 4.8. Competition between E. coli K12 (TP120A) and E. coli K12(TP120B) at $D = 0.10 \text{ h}^{-1}$ in phosphorus-limited chemostat culture. Total number of viable organisms, (●) and number of viable ampicillin resistant organisms (E.coli K12(TP120A)), (○).

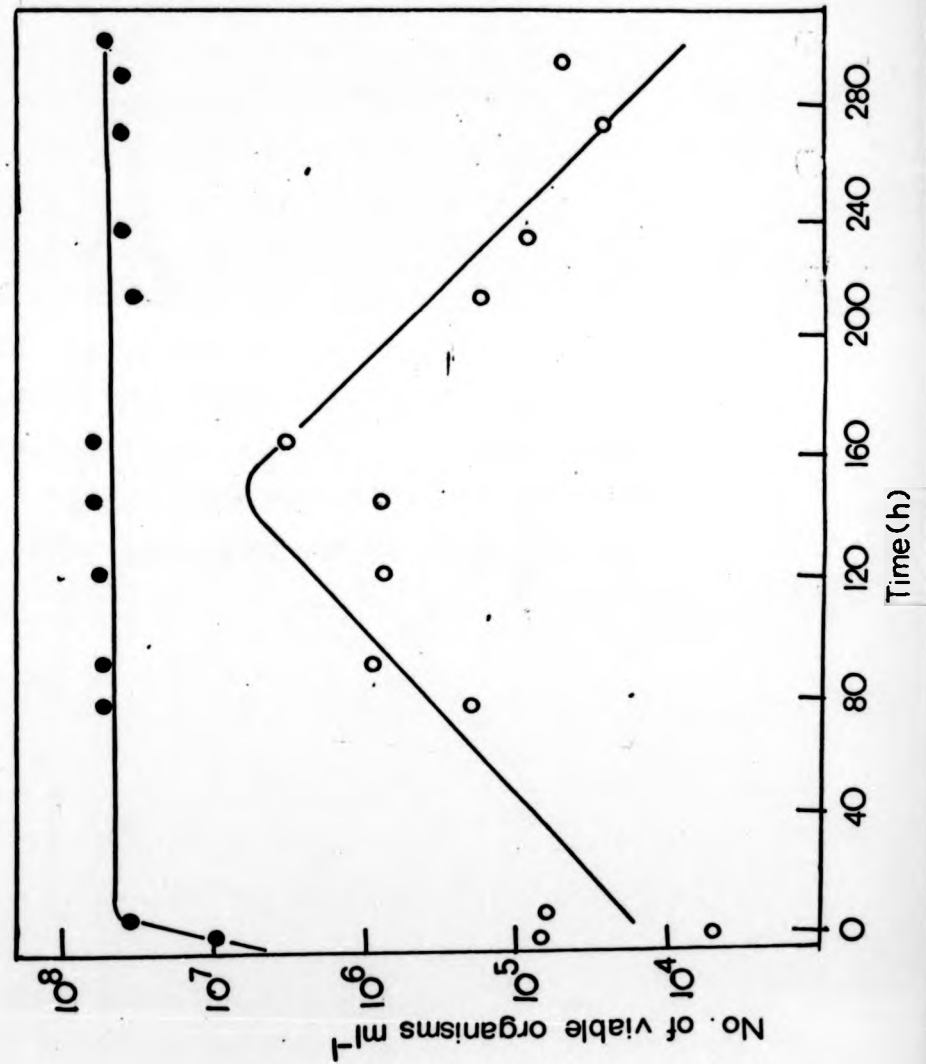
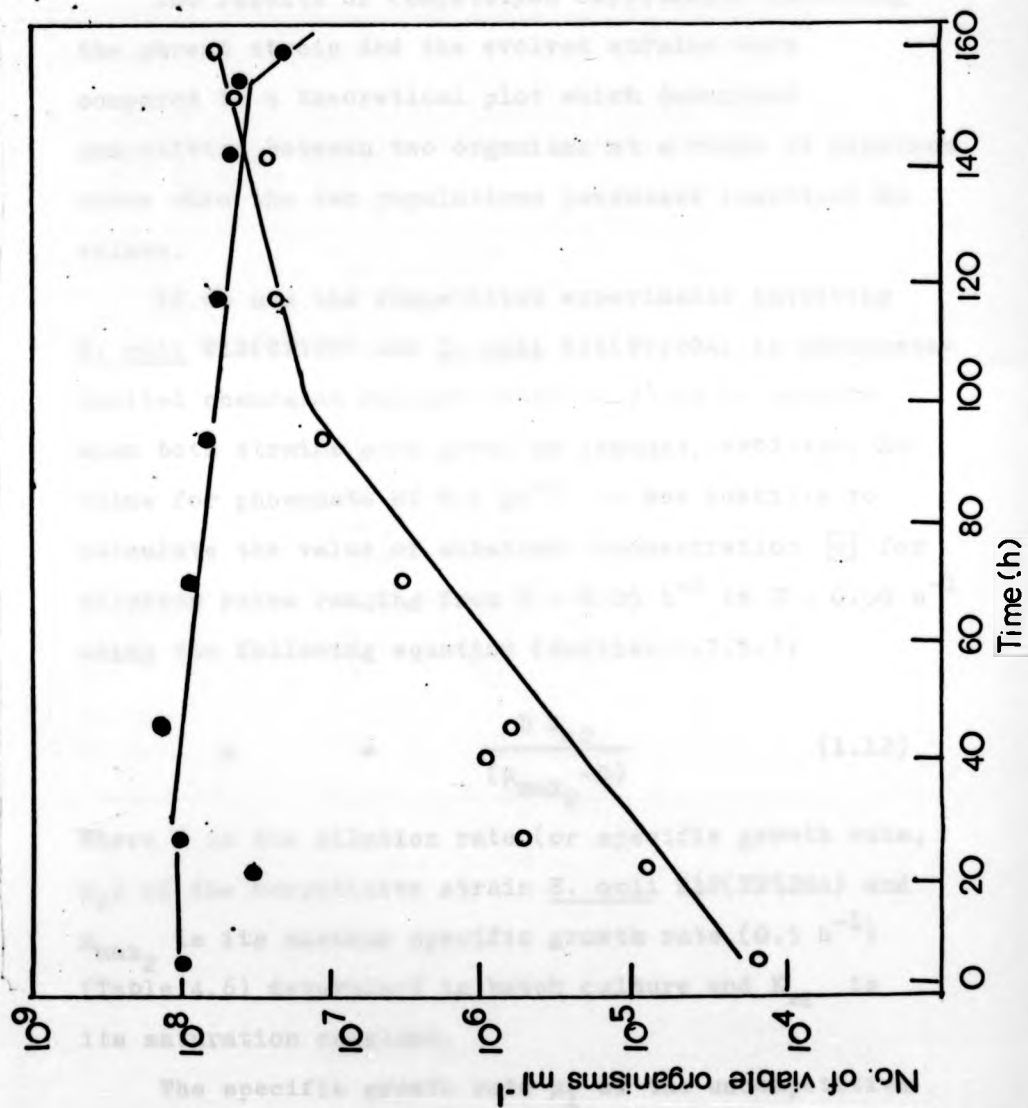


Figure 4.9 Competition between E. coli K12(TP120A) and E. coli K12(TP120D) at $D = 0.10 \text{ h}^{-1}$ in carbon-limited chemostat culture. Number of viable ampicillin resistant organisms (E. coli K12(TP120A)), (○) and number of viable(E. coli K12(TP120D)). (●).

4.5 ANALYSIS OF RESULTS

The results of competition experiments for various



The specific growth rate of the population was given by the following equation:

population was given by the following equation:

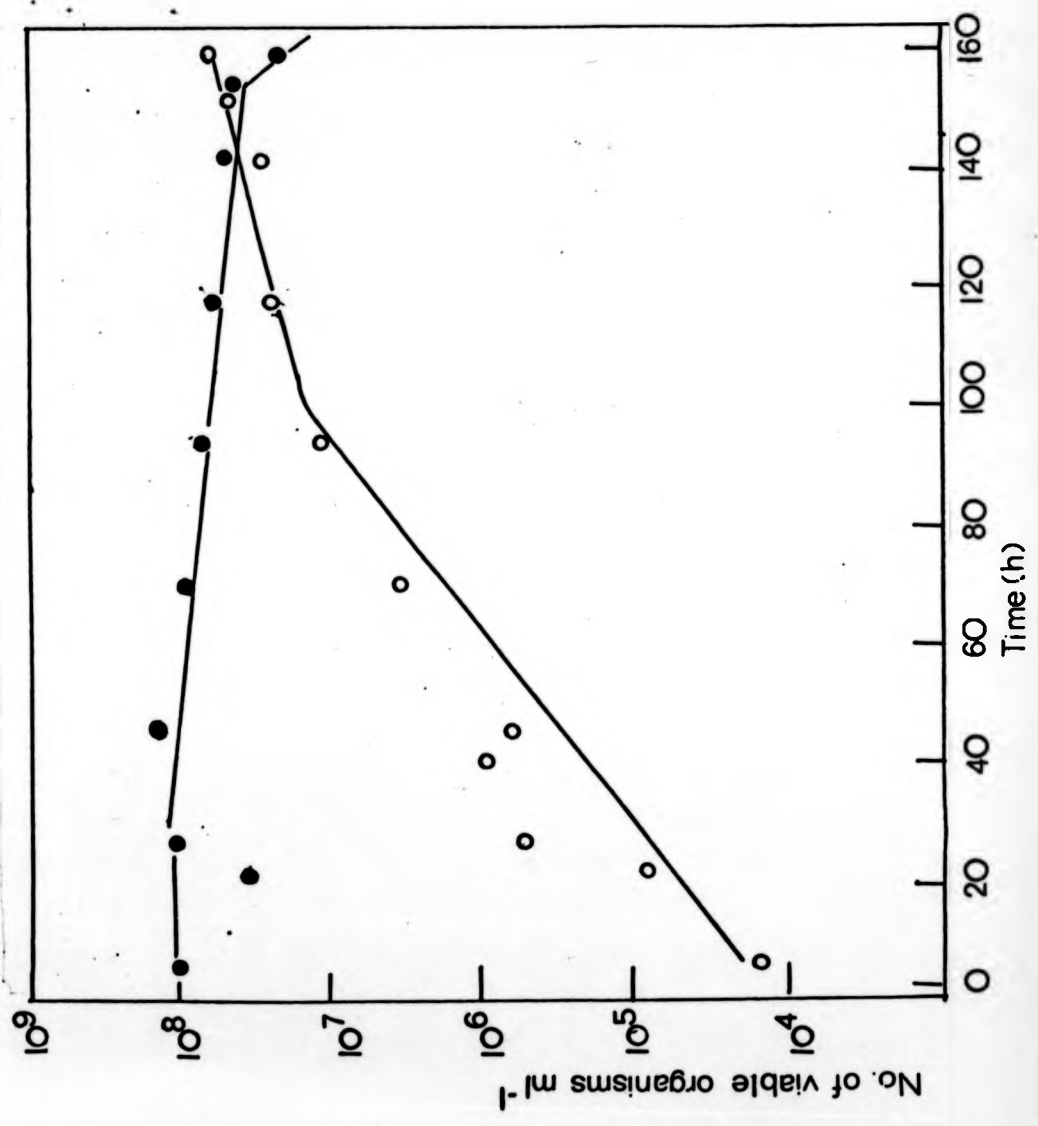
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4.5 ANALYSIS OF RESULTS

The results of competition experiments involving the parent strain and the evolved strains were compared to a theoretical plot which described competition between two organisms at a range of dilution rates when the two populations possessed identical K_s values.

If we use the competition experiments involving E. coli K12(TP120) and E. coli K12(TP120A) in phosphate-limited chemostat culture (Table 4.2) as an example when both strains were given an assumed, arbitrary K_s value for phosphate of 0.5 g l^{-1} , it was possible to calculate the value of substrate concentration $[s]$ for dilution rates ranging from $D = 0.05 \text{ h}^{-1}$ to $D = 0.50 \text{ h}^{-1}$ using the following equation (Section 1.7.5.):

$$s = \frac{D K_{s2}}{(\mu_{\max 2} - D)} \quad (1.12)$$

Where D is the dilution rate (or specific growth rate, μ_2) of the competitive strain E. coli K12(TP120A) and $\mu_{\max 2}$ is its maximum specific growth rate (0.5 h^{-1}) (Table 4.6) determined in batch culture and K_{s2} is its saturation constant.

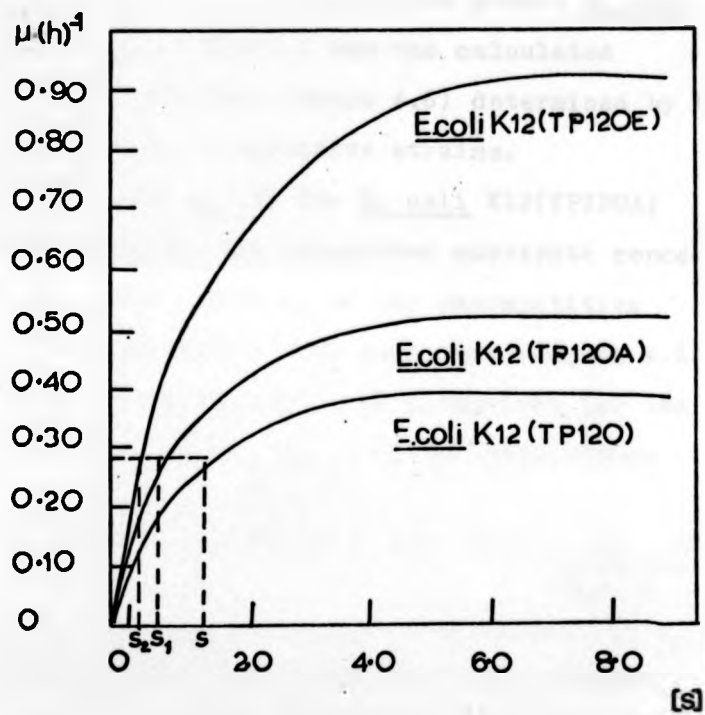
The specific growth rate μ_1 of the uncompetitive population was given by the following equation:

Table 4.6 The affect of dilution rate on substrate concentration for the organism E. coli K12(TP120A) with an assumed arbitrary K_s value for phosphate of 0.5 g l^{-1}

a - denotes dilution rates examined in two membered competition studies between E. coli (TP120) and E. coli (TP120A)

Dilution rate (D) (h^{-1})	Substrate concentration(s)	Specific growth rate of uncompetitive population (μ_1) (h^{-1})
0.05	0.055	-0.039
0.10 a	0.125	0.077
0.15 a	0.214	0.116
0.20	0.33	0.155
0.25 a	0.50	0.195
0.30	0.75	0.234
0.35 a	1.16	0.272
0.40	2.0	0.312
0.45	4.5	0.351
0.50	∞	-

Figure 4.10 A theoretical representation of the effect of specific growth rate μ on substrate concentration $[s]$ for the organisms E. coli K12 (TP120); E. coli K12(TP120A) and E. coli K12 (TP120E) (no plasmid).



$$\mu_1 = \frac{\mu_{\max 1} \cdot s}{(K_{s1} + s)} \quad (4.1)$$

assuming that $K_{s2} = K_{s1}$ and where $\mu_{\max 1}$ was the maximum specific growth of the uncompetitive strain E. coli Kl2(TPl20) (0.39 h^{-1}) and s was the calculated substrate concentrations (Table 4.6) determined by K_{s2} $\mu_{\max 2}$ values of the competitive strains.

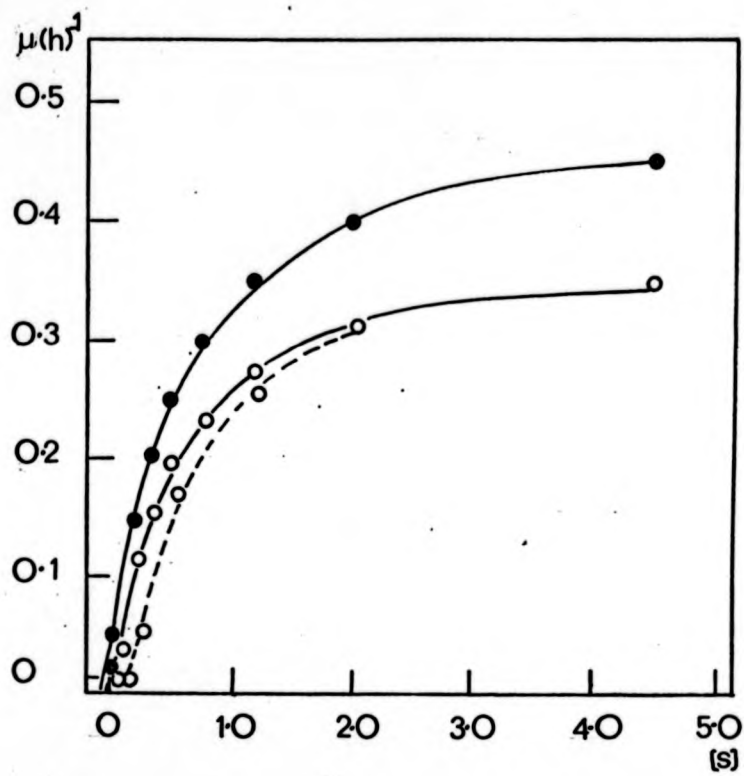
The values of μ_2 (D) for E. coli Kl2(TPl20A) were plotted against the calculated substrate concentration. The specific growth rate μ_1 of the uncompetitive population was plotted on the same axis (Figure 4.11). If instead of plotting values of μ_2 against $[s]$ the "relative" growth rate $\frac{\mu_2}{\mu_{\max 2}}$ of the competitive population and the "relative" growth rate $\frac{\mu_1}{\mu_{\max 1}}$ of the uncompetitive population was substituted for μ_1 then the two curves became superimposed on one another (Figure 4.12). This was because for the competitive population E. coli Kl2(TPl20A)

$$\mu_2 = \frac{\mu_{\max 2} \cdot s}{(K_{s2} + s)}$$

and following on

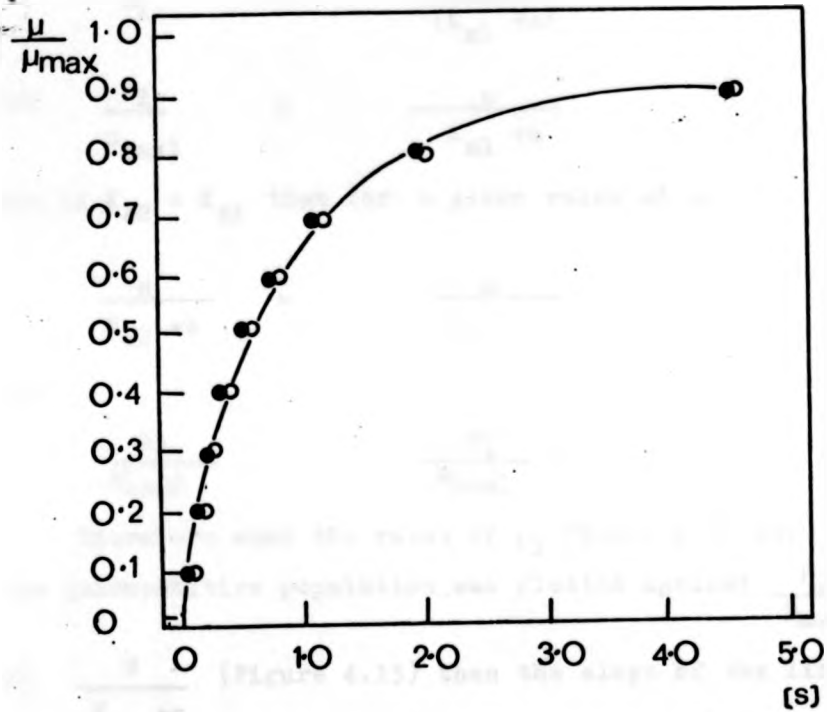
$$\frac{\mu_2}{\mu_{\max 2}} = \frac{s}{K_{s2} + s} \quad (4.2)$$

Figure 4.11 The effect of substrate concentration $[s]$ on the specific growth rates of E. coli K12(TP120), μ_1 (o) and E. coli K12(TP120A), μ_2 (●), with both having an assumed, arbitrary K_s value of 0.5 g l^{-1} . The dotted line indicated observed results and the unbroken line theoretical μ_1 values for E. coli K12(TP120).



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Figure 4.12. Data from Figure 4.12 replotted for
"relative" growth rate values for E. coli K12
(TP120A) and E. coli K12(TP120)



and similarly for the uncompetitive population
E. coli K12(TP120)

$$\mu_1 = \frac{\mu_{\max 1} \cdot s}{(K_{s1} + s)}$$

$$\text{and } \frac{\mu_1}{\mu_{\max 1}} = \frac{s}{K_{s1} + s}$$

but if $K_{s2} = K_{s1}$ then for a given value of s

$$\frac{s}{K_{s2} + s} = \frac{s}{K_{s1} + s}$$

and

$$\frac{\mu_2}{\mu_{\max 2}} = \frac{\mu_1}{\mu_{\max 1}}$$

Therefore when the value of μ_1 (Table 4.7) for the uncompetitive population, was plotted against $\frac{\mu_2}{\mu_{\max 2}}$

or $\frac{s}{K_s + s}$ (Figure 4.13) then the slope of the line

which passes through the origin, gives the maximum specific growth rate ($\mu_{\max 1}$) of E. coli K12(TP120) since:

$$\begin{aligned} \frac{\mu_2}{\mu_{\max 2}} &= \frac{\mu_1}{\mu_{\max 1}} \\ \mu_1 &= \frac{\mu_2}{\mu_{\max 2}} \cdot \mu_{\max 1} \quad (4.3) \end{aligned}$$

Table 4.7 The effect of "relative" growth rates on substrate concentration for the organism E. coli K12(Tp120) and E. coli K12(Tp120A) with assumed K_s values for phosphate of 0.5 g l^{-1} .
 a: where $\mu_{\max 2} = 0.50 \text{ h}^{-1}$ and $\mu_{\max 1} = 0.39 \text{ h}^{-1}$.

Substrate concentration (s)	Specific growth rate μ_2 of <u>E. coli K12 (Tp120A)</u> (h^{-1})	"Relative" growth rate $\mu_2/\mu_{\max 2}$ a <u>E. coli K12 (Tp120A)</u> (h^{-1})	Specific growth rate μ_1 of <u>E. coli K12 (Tp120)</u> (h^{-1})	"Relative" growth rate $\mu_1/\mu_{\max 1}$ a <u>E. coli K12 (Tp120)</u> (h^{-1})	Observed μ_1 of <u>E. coli K12 (Tp120)</u> (h^{-1})
0.055	0.05	0.10	0.039	0.10	-
0.125	0.10	0.20	0.077	0.20	0.01
0.214	0.15	0.30	0.12	0.28	0.03
0.33	0.20	0.40	0.15	0.38	-
0.50	0.25	0.50	0.19	0.49	0.16
0.75	0.30	0.60	0.23	0.59	-
0.16	0.35	0.70	0.27	0.69	0.23
2.0	0.40	0.80	0.31	0.79	-
4.5	0.45	0.90	0.35	0.89	-
	0.50	1.0	0.40	1.0	-

Using these equations the $\mu_{\max 1}$ value of the uncompetitive population E. coli K12(TP120) was calculated to have the theoretical value of 0.39 h^{-1} (Figure 4.13). However the experimentally determined value of μ_1 for E. coli K12(TP120) did not fall on the theoretical line at lower values of $\frac{\mu_2}{\mu_{\max 2}}$ (Figure 4.13). However Figure 4.14 illustrates a theoretical situation where E. coli K12(TP120) was given an assumed K_{s1} of 0.75 g l^{-1} whilst E. coli K12(TP120A) still had the K_{s2} value of 0.5 g l^{-1} , such that $K_{s1} > K_{s2}$. The plot of μ_1 against $\mu_2/\mu_{\max 2}$ now described a curve, which was lower than the straight line plot in Figure 4.13 when the organisms had equal K_s values. The curve is produced if $K_{s1} > K_{s2}$ since from equation 4.2

$$\frac{\mu_2}{\mu_{\max 2}} = \frac{s}{(K_{s2} + s)} \quad \text{and} \quad \frac{\mu_1}{\mu_{\max 1}} = \frac{s}{(K_{s1} + s)}$$

$$\therefore \mu_1 = \frac{\mu_2}{\mu_{\max 2}} \left[\frac{\mu_{\max 1} (K_{s2} + s)}{(K_{s1} + s)} \right]$$

This has the same form as equation 4.3 but now the slope is not constant because s depends on the value of μ_2 and when $K_{s1} > K_{s2}$ the result is a curve as shown in Figure 4.14. It can be seen that the experimental values fitted more closely to this second theoretical plot. This demonstrated that the successful evolved strain had a lower K_s value than

Figure 4.13 The effect of "relative" growth rate $\frac{\mu_2}{\mu_{\max 2}}$ of the competitive strain E. coli K12(TP120A) on the specific growth rate μ_1 of E. coli K12(TP120) when both have an arbitrary, assumed K_s value of 0.5 g l^{-1} (● denotes the theoretical values and ○ observed values).

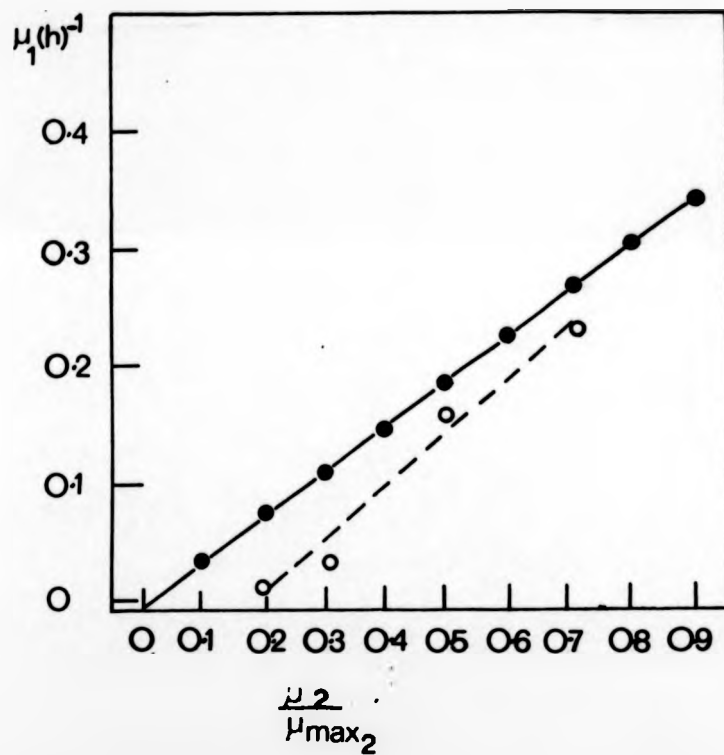
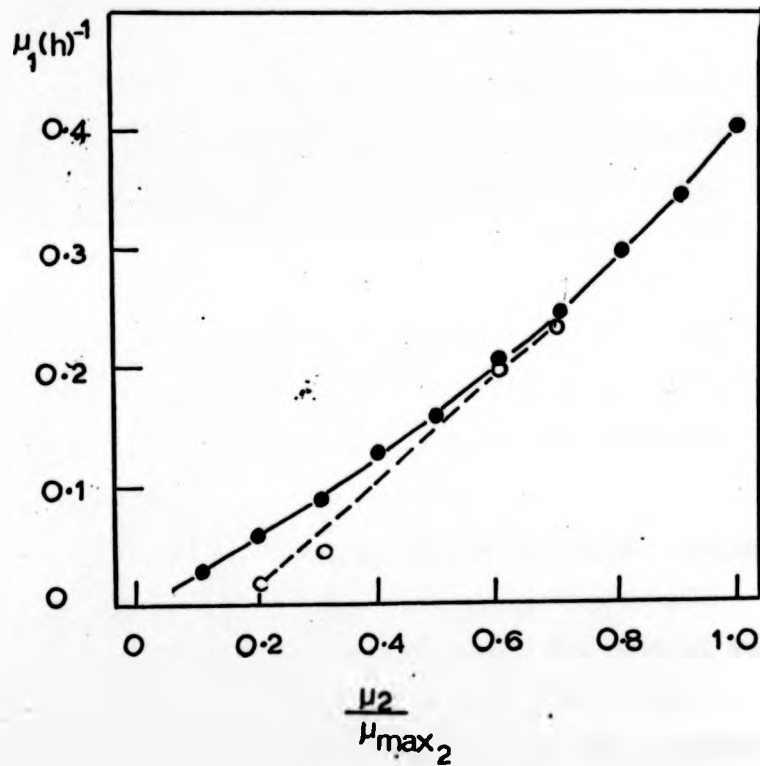


Figure 4.14 The effect of the "relative" growth
 μ_2 of the competitive strain E. coli K12(TP120A)
 μ_{max_2}
 with an assumed K_s value of 0.5 g l^{-1} on the specific
 growth rate (μ_1) of E. coli K12(TP120) with a K_s
 value of 0.75 g l^{-1} , (\bullet denotes theoretical values
 and \circ observed values).



the parent strain.

The same calculations were applied for the uncompetitive strain E. coli K12(TP120) in competition with E. coli K12(TP120B), E. coli K12(TP120D) and E. coli K12(TP120E).

Figure 4.15 showed that in competition with E. coli K12(TP120B) the observed results produced a line which passed through the origin with a slope which gave a μ_{max1} value of 0.24 h^{-1} and was below the steeper theoretical slope. This implied that the evolved strain was more competitive than the simple difference in μ_{max} values suggested and indicated that E. coli K12(TP120B) had a K_s value lower than the parent strain.

In competition with E. coli K12(TP120D) (Figure 4.16) the observed values of μ_1 for E. coli K12(TP120) were again lower than the predicted, theoretical values based on differences in μ_{max} values only. This suggested that the evolved strain also had a greater affinity for the substrate (lower K_s value) than the uncompetitive population. The difference in theoretical and observed values were so great and the evolved strain was so much more competitive than expected from μ_{max} batch values, the organism must have undergone mutations which have resulted in a very competitive strain.

Figure 4.15 The effect of relative growth rate

$\frac{\mu_2}{\mu_{\max_2}}$ of the competitive strain E. coli K12(TP120B) on the specific growth rate (μ_1) of E. coli K12(TP120) when both have an assumed, arbitrary K_s value of 0.5 g l^{-1} (● denotes theoretical values and ○ observed values).

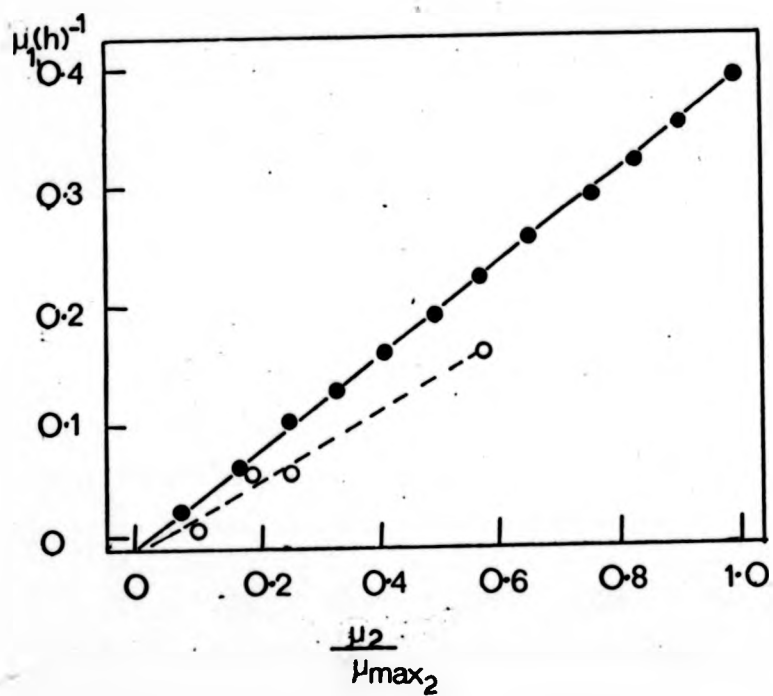
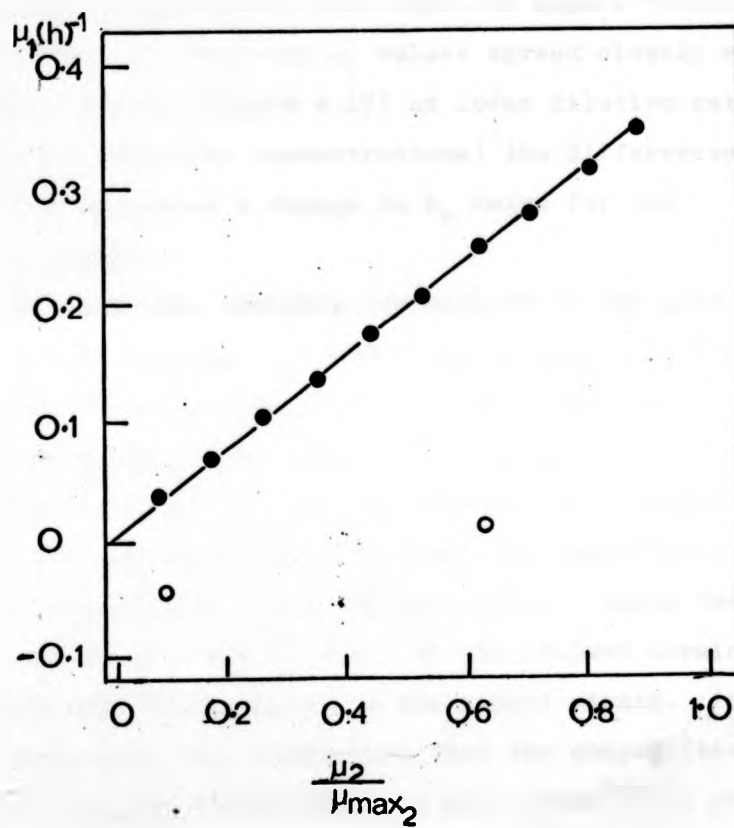


Figure 4.16. The effect of "relative" growth rate $\frac{\mu_2}{\mu_{\max_2}}$ of the competitive strain E. coli K12(TP120D) and the specific growth rate (μ_1) of E. coli K12(TP120) when both have assumed, arbitrary K_s value of 0.5 g l^{-1} (● denotes theoretical values and ○ observed values.).



The observed results for competition experiments involving E. coli K12(TP120) and E. coli K12(TP120E) (no plasmid) again showed that while at higher "relative" growth rates the observed μ_1 values agreed closely with predicted values (Figure 4.17) at lower dilution rates (and lower substrate concentrations) the differences in μ_1 values suggested a change in K_s value for the evolved strain.

When the same analysis was applied to the pair of organisms E. coli K12(TP120) and E. coli K12(TP120A) competing in carbon-limited conditions using an arbitrary K_s value for glucose of 0.05 g l^{-1} (Table 4.2), the observed μ_1 values for the uncompetitive strain fitted to a straight line but which was again lower than the the theoretical curve (Figure 4.18). Again the slope showed that the K_s value of the evolved strain had been modified compared to the parent strain. It also emphasised the observation that the competitive organism E. coli K12(TP120A) was more competitive in phosphorus-limited conditions than carbon-limited conditions

The advantage of treating the results from chemostat competition experiments in this manner was two-fold since

- (1) It was possible to predict whether or not the K_s values of parent and evolved strains were the same.

Figure 4.17. The effect of "relative" growth rate $\frac{\mu_2}{\mu_{\max 2}}$ of the competitive strain E. coli K12(TP120E) (no plasmid) on the specific growth rate μ_1 of E. coli K12(TP120) when both have an assumed, arbitrary K_s value of 0.5 g l^{-1} (● denotes theoretical values and ○ observed values.).

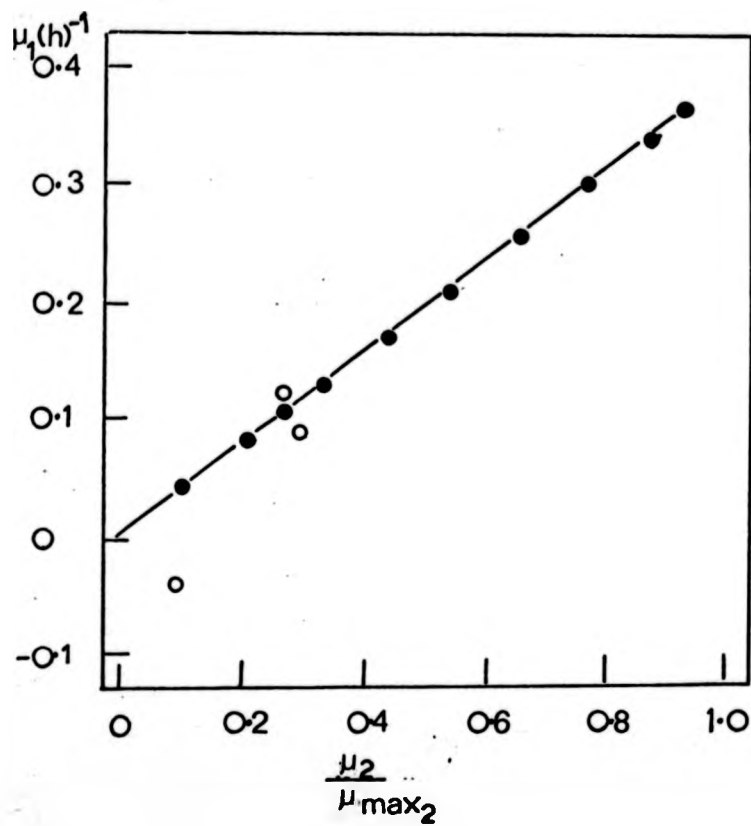
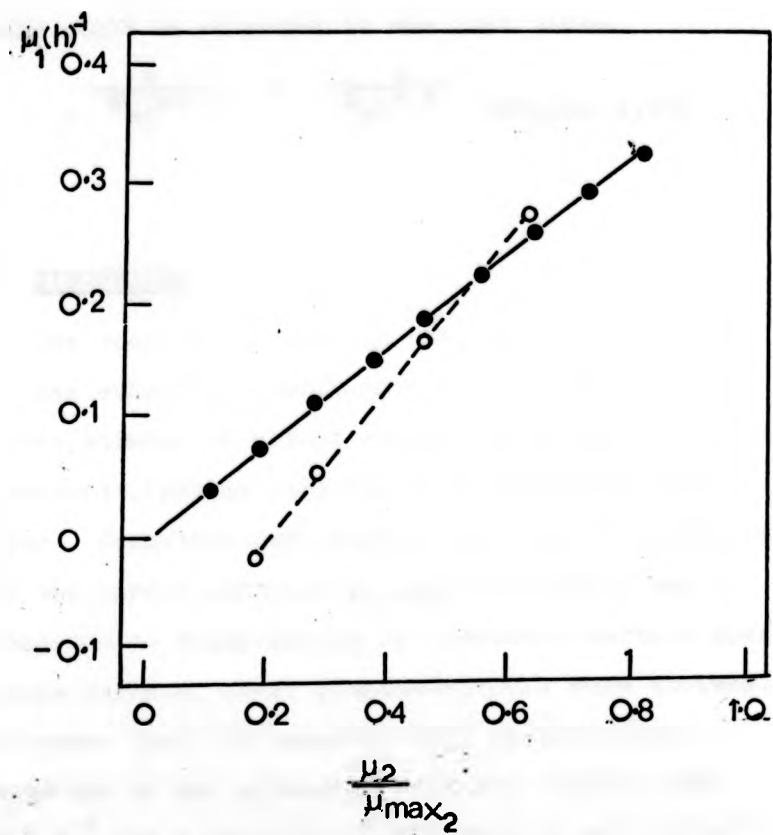


Figure 4.18 The effect of the "relative" growth rate $\frac{\mu_2}{\mu_{\max 2}}$ of the competitive strain E. coli K12(TP120A) on the specific growth rate of E. coli K12(TP120) in carbon-limited chemostat culture when both have an assumed, arbitrary K_s value of 0.5 g l^{-1} (● denotes theoretical values and ○ observed values).



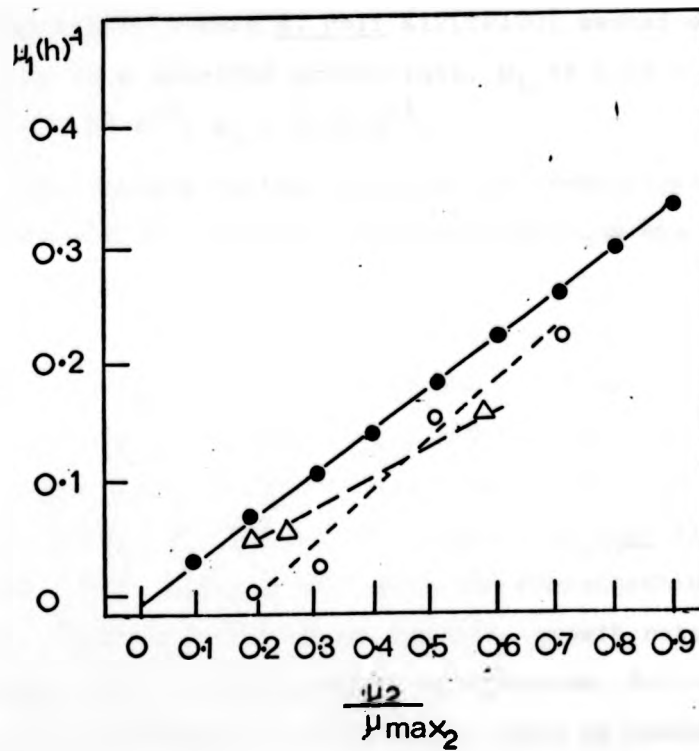
(2) The results from experiments involving different competitive strains but the same uncompetitive strain could be compared in one plot since

$$\frac{s}{K_{s2} + s} = \frac{s}{K_{s1} + s} \quad (\text{Figure 4.19})$$

4.6 DISCUSSION

The results reported in this section confirmed that the selective growth advantages shown by evolved strains in closed culture were also significant in nutrient-limited conditions at submaximal growth rates. Selection experiments (Section 3) indicated that the parent organism E. coli K12(TP120) was at a competitive disadvantage in chemostat culture when strains carrying fewer plasmid-markers were evolved. This meant that the uncompetitive parent strain washed out of the chemostat at rates ranging from -0.012 h^{-1} (at a specific growth rate μ_1 of 0.33 h^{-1}) when at a dilution rate of $D = 0.35 \text{ h}^{-1}$ to a rate of -0.07 h^{-1} ($\mu_1 = 0.03 \text{ h}^{-1}$) at a dilution rate of 0.10 h^{-1} in phosphorus-limited conditions. These washout rates and specific growth rate values were very similar to those observed for the uncompetitive parent strain E. coli K12(TP120) in the individual mixed culture experiments

Figure 4.19. A comparison of the effect of "relative" growth rate $\frac{\mu_2}{\mu_{\max 2}}$ of the competitive strains E. coli K12(TP120A) (\circ) and E. coli K12(TP120B) (Δ) on the specific growth rate μ_1 of E. coli K12(TP120) in phosphate-limited conditions when all strains have assumed, arbitrary K_s values of 0.5 g l^{-1} (● denotes the theoretical values of μ_1 .)



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with evolved strains (Tables 4.2 to 4.5). For example, when in competition with E. coli K12(TP120A) at $D = 0.10 \text{ h}^{-1}$ in phosphorus-limited conditions the uncompetitive strain E. coli K12(TP120) washed out while growing at a specific growth rate, μ_1 of 0.02 h^{-1} and at $D = 0.35 \text{ h}^{-1}$, $\mu_1 = 0.23 \text{ h}^{-1}$.

The results in this section also demonstrated (Tables 4.2 to 4.5) that the organisms with the higher μ_{max} values always had the greater competitive advantage. This meant that the specific growth rate, μ_1 of the parent strain was reduced more when in competition with an organism with a higher μ_{max} value. This can be explained theoretically by the observation that a more rapidly growing organism, for example E. coli K12(TP120E) (Figure 4.10) reduced the substrate concentration to a lower value (s_2) at a given specific growth rate compared with a less competitive organism, for example E. coli K12(TP120A). This means that in competition experiments between E. coli K12(TP120) and E. coli K12(TP120E) the parent organism is unable to sustain a high enough growth rate when the substrate concentration is reduced to s_2 and so washes out of the chemostat. In turn at the same growth rate, growth of E. coli K12(TP120A) results in a substrate concentration s_1 .

(Figure 4.10) which means that the parent organism was again unable to sustain a high enough growth rate and washes out. However the growth rate of the uncompetitive population, μ_1 is greater at s_1 than at s_2 .

The results from selection experiments and batch growth determinations predicted that the reduction in plasmid DNA resulted in a growth advantage for the evolved strain by increasing their μ_{\max} values. However, analysis of results from competitive experiments (Section 4.5) indicated that differences in μ_{\max} values alone could not account for the competitiveness of the evolved strains. Instead Figures 4.13 and 4.17 indicated that the competitive strains had also evolved a greater affinity for the growth-limiting substrate, that is, had a lower K_s values than the parent strain.

The same situation was found for all evolved strains examined but was particularly marked in the case of E. coli K12(TP120D). This strain was far more competitive than batch maximum specific growth rate determination (Section 3.5. Table 3.8) suggested. It appeared that this organism must have evolved a more efficient assimilation or metabolism of substrate than the parent strain, as a result of nutrient-limited growth so that its growth advantage was not due simply

to a reduction in DNA.

Therefore, whilst the competition experiments provided additional evidence for the theory that the reduction in plasmid DNA in evolved strains resulted in increased specific growth rates which were a contributory advantage during competition under restricted growth conditions the situation was more complex than indicated previously.

Auxotrophic mutants of Bacillus subtilis described by Zamenhof and Eichorn (1967) and E. coli auxotrophs investigated by Mason and Slater (1979) have demonstrated increased growth efficiency compared to prototrophic strains. Also organisms carrying plasmid cloning vehicles which are present in high copy numbers during normal growth and thus can be used to produce high yields of selected gene products are rapidly outgrown by plasmid-minus organisms and lost from a population unless a positive selective pressure is applied (Hershfield et al. 1974). Lin et al. (1977) demonstrated that this principle was even extended to λ lysogens of E. coli strains which had a growth rate advantage over non-lysogenic strains when grown in glucose limited aerobic conditions.

The failure of plasmid-carrying strains in competition with plasmid-minus strains has been previously reported by Melling et al. (1976), Wouters and van Andel (1979) and Adams et al. (1979) for continuous culture

experiments and also in repeated subculture experiments by Dale and Smith (1979). Melling et al. (1976) found that while the RPl - minus strain was always successful under phosphate-limited conditions, in carbon-limited conditions the outcome depended on the ratio of the plasmid-carrying to isogenic plasmid-minus strain which showed that for RPl phosphate-limitation was a stronger selection pressure. E. coli K12(TP120) is similarly less competitive in phosphate-limited conditions. Wouters et al. (1979) described plasmid-minus E. coli K12 take over in all limitations examined and even in anaerobic conditions but gave no indication as to which limitation produced the greatest competitive effect. In all of the chemostat culture experiments reported (Melling et al. (1976) Wouters et al., (1979) and Adams et al., (1979)) the competitive effect of the plasmid-minus strain was attributed simply to an increased μ_{\max} value compared to an isogenic plasmid-minus and K_s values of the organisms were not measured.

It is perhaps not surprising that the evolved strains in this study had apparently altered K_s values compared to the parent strain since they had been evolved under nutrient-limited conditions and had lost drug resistance functions, some of which are membrane

associated. E. coli K12(TP120A), E. coli K12(TP120D) and E. coli K12(TP120E) (no plasmid) had all lost tetracycline resistance which is known to be a membrane associated resistance mechanism. Tetracycline resistant and sensitive strains have been shown to have differences in the uptake of the antibiotic (Levy et al. 1974; Robertson and Reeve, 1972). It has been suggested that the plasmid codes for an altered tetracycline uptake system which switches off the tetracycline uptake system of the host organism. This process may involve a modification of other associated assimilation systems, possibly by changes in membrane structure and therefore influences nutrient uptake resulting in changed K_s values in evolved, sensitive strains. In this study involving the plasmid TP120 the affinity for phosphate seemed more affected (Figures 4.11 to 4.17), but the relationship between the two uptake mechanisms is unknown and would require further investigation. Assimilation of carbon and phosphate was examined briefly in preliminary radiochemical experiments described in Section 6 but the results produced were rather inconclusive. A study of the membrane structure of the parent and evolved strains ought to provide useful information. Finch and Brown (1975) described the effect of nutrient limitation on the

resistance of P.aeruginosa to EDTA and polymixin and showed that the change in sensitivity was due to a change in structure and properties of the cell envelope. Therefore cell membrane studies accompanied by accurate measurements of the K_s values for the evolved strains should be carried out.

The mixed culture competition experiment suggested that the evolved strains are at a greater advantage at lower dilution (growth) rates and lower substrate concentrations (Table 4.2., 4.3 and 4.5). These conditions are more typical of several situations in Nature (Brown, 1977) so that plasmid-carrying strains may be at greater disadvantages than batch culture experiments would indicate. The exact nature of conditions found in natural situations has not been well documented, especially in terms of nutrient limitation but there has been evidence to suggest that bacterial doubling times in Nature are very slow and probably in the order of 20 to 24 h (Meynell and Subbiah, 1963; Eudy and Burroughs, 1973).

Conversely some plasmid-carrying strains may have some advantage in in vivo situations, for example, in serum where iron is thought to be the growth limiting nutrient (Bullen, Rogers and Griffiths, 1974) the iron uptake was increased by the presence of some plasmids, e.g. Col V, in their bacterial hosts (Hardy, 1975; Williams, P.H. 1977). Pathogenicity and survival of

the host strain were increased whilst the plasmids-coded colicin producing function was apparently unaffected. Since plasmid-minus organisms were not frequently isolated in selection experiments (Section 3) it could suggest that whilst the plasmid dispenses with "unwanted" drug resistance functions it maintains other undetermined functions on the plasmids and these could even be involved in the increased specific growth rates shown by evolved strains. There is evidence from other organisms that the resistance genes have an influence on host cell metabolism. For example the penicillinase gene present in Staphylococcus epidermidis has been found to not only code for resistance but is also involved in regulation of mannitol uptake, ribose fermentation and phospho - β glucosidase activity. It is also thought to affect phage adsorption, restriction and modification, as well as modifying growth factor requirements (Shaeffler, 1972). Loss of such a gene would apparently be immediately recognised by a reversion to antibiotic sensitivity but would have other more complex consequences for the host organism.

Whilst attempting to extrapolate mixed culture chemostat competition results to natural situations it is important to recognise the limitation of the experimental procedure. For example, it was found that while the ratio of the two competing populations present at the onset of media flow did not apparently influence the outcome of competition, the inoculation procedure did (Table 4.1) This data possibly indicates that the difference in

competitiveness found when the non-simultaneous inoculation procedure was used was due to the difference in physiological state of the two populations. This may be more variable in a natural situation and so competition would be further complicated. The observation that inoculation procedure influenced competition also makes it difficult to compare results reporting the behaviour of different plasmid-carrying strains because of the varied inoculation procedures adopted by different groups of workers. The most efficient inoculation procedure ought to involve the inoculation of the two populations from two separately growing steady-state cultures into a single chemostat. Hopefully the simultaneous inoculation method using batch culture inocula provided an acceptable compromise. Adams et al. (1979) using chemostat grown inocula found that organisms carrying plasmids were successful if their initial frequency was above 50%. Since, in that study, the plasmid-carrying organism produced a plasmid-coded colicin which has a direct competitive effect on non plasmid-carrying organisms the same principle cannot be directly related to drug-resistance plasmids. In fact, the authors make the point that this critical frequency for a population would undoubtedly change for different plasmids in different environments and it would appear that for E. coli K12(TP120) it would have a very high value to result in selection of the

plasmid-carrying strain.

Another disadvantage in the design of mixed population experiments described in this section with the parent strain E. coli K12(TP120) and evolved strains was that it was only possible to follow growth of the parent strain directly due to a lack of additional markers on the evolved strains. However in these experiments and, in competition experiments between evolved strains an initial increase in the organism destined to wash out of the chemostat vessel was observed. This observation has also been described by Meers (1973) for an experiment with Aerobacter aerogenes and Torula utilis in a phosphate-limited chemostat. The fluctuating period of growth prior to washout of the non-competitive population was also apparently found by Wouters *et al.*, (1979) who reported that washout did not begin until 50 h after inoculation of a competitive population. This period may be longer for other pairs of competitors and could provide an explanation for the observation that in some studies there has been a failure to report competition between the plasmid-carrying and plasmid-minus strains or in selection experiments, the evolution of plasmid-minus strains.

5. A VIABILITY STUDY OF THE PLASMID-CARRYING ORGANISM
E. COLI K12(TP120) AND THE PLASMID-MINUS ORGANISM
E. COLI K12(TP120E) (NO PLASMID) IN PHOSPHORUS-LIMITED
CONTINUOUS-FLOW CULTURE

The results of batch culture μ_{\max} determinations and chemostat competition studies showed that evolved strains of E. coli K12 with fewer drug resistance markers than E. coli K12(TP120) grew more rapidly than the parent strain E. coli K12(TP120) under nutrient rich and nutrient-limited conditions. The most successful organism was the plasmid-minus strain E. coli K12(TP120E) (no plasmid). The growth of the parent strain E. coli K12(TP120) and the most successful evolved strain was therefore compared in separate chemostat cultures growing in phosphorus limitation at $D = 0.10 \text{ h}^{-1}$ and $D = 0.35 \text{ h}^{-1}$ to determine if it was possible to detect any major differences in growth characteristics which could be responsible for the slower growth rate of the parent strain. The growth of E. coli K12(TP120E) (no plasmid) was also examined at the additional growth rates of $D = 0.20 \text{ h}^{-1}$ and $D = 0.60 \text{ h}^{-1}$. In Section 4.5 it appeared that the "relative" growth rate of an organism, that is, the percentage of its maximum specific growth rate that an organism grew at was the important factor in determining the outcome of competition. Using the "relative" growth rate rather than the absolute specific growth rate values, the growth of different organisms could also be compared (Section 4.5). At $D = 0.10 \text{ h}^{-1}$

E. coli K12(TP120) was growing at 25% of its μ_{\max} value whilst E. coli K12(TP120E) was growing at only 11% of its maximum specific growth rate of 0.90 h^{-1} (Table 5.1). Therefore the growth of E. coli K12(TP120E) (no plasmid) was also examined at $D = 0.20 \text{ h}^{-1}$ which is 33% of its μ_{\max} value. Similarly at $D = 0.35 \text{ h}^{-1}$ the parent organism was growing at 87% of its maximum specific growth rate while this was only 38% of the μ_{\max} value of E. coli K12(TP120E). The viability and growth characteristics of E. coli K12 (TP120E) were examined at $D = 0.60 \text{ h}^{-1}$ which was 66% of its maximum specific growth rate. The dilution rate of $D = 0.78 \text{ h}^{-1}$ which was 87 % of the μ_{\max} value for E.coli K12(TP120E) was too difficult to maintain with the growth system used. Therefore the growth of the parent and evolved strain was examined and compared for these approximately comparable values.

5.1. THE EFFECT OF DILUTION RATE ON BIOMASS

Table 5.1 shows that the biomass of the parent and evolved strain as represented by absorbance and dry weight determinations decreased with an increasing growth rate. Total carbon measurements however increased for the parent strain from 20 ppm at $D = 0.10 \text{ h}^{-1}$ to 25 ppm at $D = 0.35 \text{ h}^{-1}$. For the evolved strain E. coli K12(TP120E) (no plasmid) the total carbon measurements were lower at $D = 0.35 \text{ h}^{-1}$ compared to $D = 0.10 \text{ h}^{-1}$. The general trend of the effect of growth rate on biomass measurements for the two organisms indicated that the parent strain E. coli

Table 5.1. The effect of dilution rate on biomass of E. coli K12(TP120) and E. coli K12(TP120E) (no plasmid)

Organism	Dilution rate (h ⁻¹)	Percentage of μ_{max}	Absorbance (520 nm)	Dry Weight ($\mu\text{g ml}^{-1}$)	Total Carbon (ppm)
<u>E. coli</u> K12(TP120)	0.10	25	0.19	1.56	20
	0.35	87	0.16	0.90	25
<u>E. coli</u> K12(TP120E) (no plasmid)	0.10	11	0.17	1.01	30
	0.20	33	0.16	0.90	55
	0.35	38	0.14	0.99	18
	0.60	66	0.09	0.10	N.D.

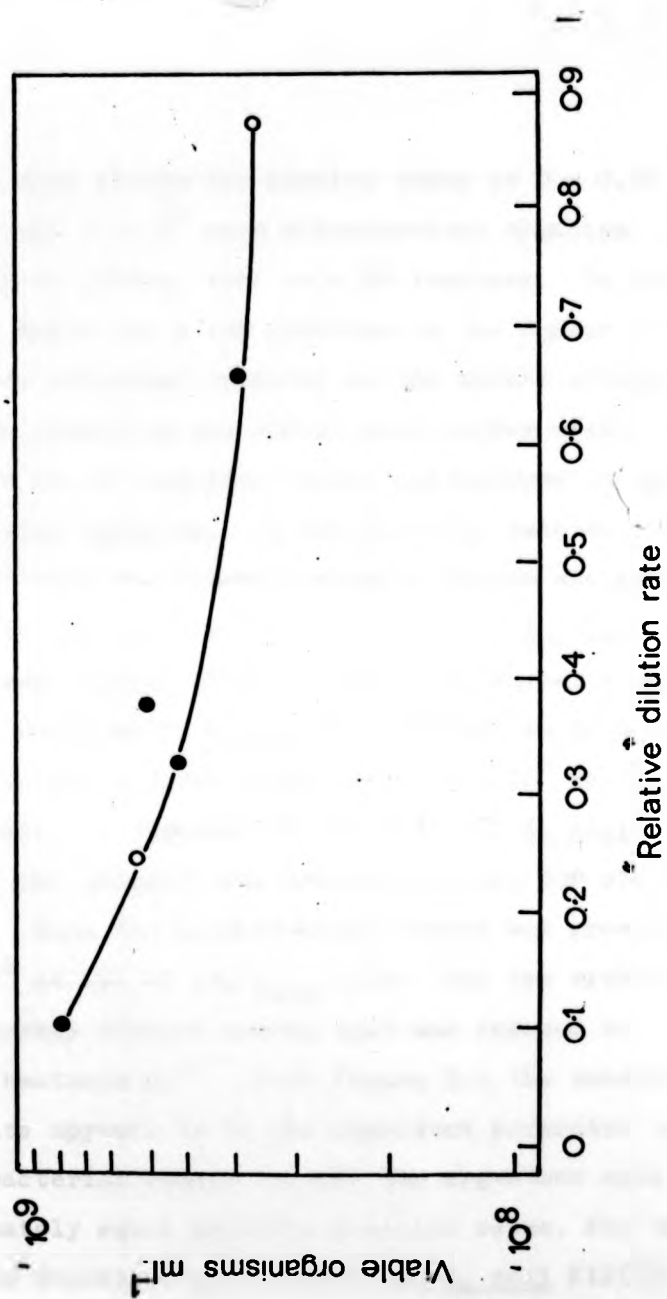
K12(TP120) was in fact growing better for the same "relative" growth rate than the evolved strain. At $D = 0.35 \text{ h}^{-1}$ when the parent organism was growing at 87% of its μ_{max} value (Table 5.1) it had higher absorbance and total carbon measurements than the evolved strain growing at its highest relative growth rate value examined. At $D = 0.20 \text{ h}^{-1}$ when E.coli K12(TP120E) (no plasmid) was growing at 33% of its μ_{max} value it has an absorbance of 0.16 and a dry weight measurement of $0.90 \mu\text{g ml}^{-1}$ compared to the parent organism at $D = 0.10 \text{ h}^{-1}$ growing at 25% of its maximum specific growth rate having an absorbance of 0.19 and a dry weight of $1.5 \mu\text{g ml}^{-1}$ which was 73% higher than that of E. coli K12(TP120E) (no plasmid).

5.2. THE EFFECT OF DILUTION RATE ON TOTAL AND VIABLE COUNTS

The viable and total counts of the parent strain E. coli K12(TP120) and the evolved strain E.coli K12(TP120E) (no plasmid) decreased with an increasing growth rate. Figure 5.1 shows how the viable count was related to the increase in "relative" growth rate for the two organisms. The plot of the percentage of the maximum specific growth rate against bacterial counts enables the effect of dilution rate for the two organisms to be compared (Section 4.5).

The total count of bacteria determined by Coulter Counter measurements reflects closely the viable counts made on Hershey defined medium agar plates. The difference in bacterial counts shown when comparing the two organisms was greater on defined agar than on nutrient agar plates.

Figure 5.1. The effect of "relative" growth rate on the viable count of organisms E. coli K12(TP120) (o) and E. coli K12(TP120E) (●) on Hershey's defined medium agar plates.



On nutrient agar plates for samples taken at $D = 0.10 \text{ h}^{-1}$ there were only 3×10^7 more plasmid-minus organisms ml^{-1} than the parent strain, that is a 4% increase. On defined agar plates there was a 44% increase in the number of plasmid-minus organisms compared to the parent strain. Overall, the plasmid-minus strain gave higher viable and total counts at all dilution rates and appeared to be the more successful organism. At the dilution rate of $D = 0.35 \text{ h}^{-1}$ when the plasmid-carrying strain was growing at 87% of its maximum specific growth rate E. coli K12(TP120) was assayed at only 3.78×10^8 bacteria ml^{-1} (Table 5.2) compared to E. coli K12(TP120E) which at the same dilution rate had a viable count of 6.07×10^8 which was 37% higher. However at $D = 0.35 \text{ h}^{-1}$ E. coli K12(TP120E) (no plasmid) was growing at only 39% of its μ_{max} value. When the plasmid-minus strain was grown at $D = 0.60 \text{ h}^{-1}$ at 66% of its μ_{max} value then the viable count on Hershey defined medium agar was reduced to 3.95×10^8 bacteria ml^{-1} . From Figure 5.1 the relative dilution rate appears to be the important parameter in determining bacterial counts for the two organisms such that at approximately equal relative dilution rates, for example, the "relative" dilution rate of 0.25 for E. coli K12(TP120) and the "relative" dilution rate of 0.33 for E. coli K12(TP120E) (no plasmid) the viable counts are very similar (Table 5.2 and Figure 5.1).

Table 5.2. The effect of dilution rate on bacterial counts of E. coli K12(TP120) and E. coli K12(TP120E) (no plasmid)

Organism	Dilution rate (h ⁻¹)	"Relative" dilution rate	Viable count bacteria ml ⁻¹			Total count bacteria ml ⁻¹ x 10 ⁸
			Nutrient agar x 10 ⁸	Hershey defined agar ⁸ x 10 ⁸		
<u>E. coli</u> K12(TP120)	0.10	0.25	7.3	6.33		6.3
	0.35	0.87	4.54	3.78		5.3
<u>E. coli</u> K12(TP120E) (no plasmid)	0.10	0.11	7.6	9.1		8.85
	0.20	0.33	4.74	5.13		5.13
	0.35	0.38	4.73	6.07		6.0
	0.60	0.66	4.68	3.95		3.0

5.3. THE EFFECT OF DILUTION RATE ON CELL VOLUME.

Cell volume measurements were found to decrease with an increase in growth rate for both of the organisms examined. At $D = 0.10 \text{ h}^{-1}$ two different cell volumes were recognised for the plasmid-minus evolved strain (Table 5.3). There was a general trend for the plasmid-carrying parent strain to have a larger cell volume than the evolved plasmid-minus strain. For E. coli K12(TP120E) (no plasmid) the cell volume when it was growing at 66% of its maximum specific growth was less than half of the cell volume of the plasmid-carrying strain growing at 87% of its μ_{max} value.

5.4. DISCUSSION

The comparison of growth characteristics of the parent strain E. coli K12(TP120) and the evolved strain E. coli K12(TP120E) (no plasmid) under the same controlled conditions produced conflicting results. Whilst biomass determinations indicated that the plasmid-carrying strain was the more successfully growing organism, bacterial counts indicated that the reverse was true. However, it must be stressed that overall the differences both in biomass determinations (Table 5.1) and viable counts (Table 5.2) were not large but these differences may have been significant when growing in mixed culture in nutrient-limited conditions (Section 4.).

Table 5.3. The effect of dilution rate on cell volume for organisms E. coli K12(TP120) and E. coli K12(TP120E) (no plasmid)

Organism	Dilution rate (h ⁻¹)	"Relative" dilution rate	Cell volume (μm) ³
<u>E. coli</u> K12(TP120)	0.10	0.25	1.786
	0.35	0.87	1.721
<u>E. coli</u> K12(TP120E) (no plasmid)	0.10	0.11	1.095
			0.807
	0.20	0.33	1.162
	0.35	0.38	1.145
	0.60	0.66	0.70

The cell volume measurements combined with biomass determinations indicated that the plasmid-carrying organism was present at slightly lower numbers but as larger cells in a steady-state culture when compared with the evolved strain which may be a smaller more rapidly dividing cell. Nordstrom, Engberg, Gustafsson, Molin and Uhlin, (1977) observed that for an E. coli K12 carrying one copy of the plasmid R1 drd - 19 or multiple copies of the plasmid pKN103, the cell dimensions increased approximately 50% in length and 20% in diameter when compared to the plasmid-minus cell. This indicated the formation of a filament-like cell and it was concluded that in the plasmid-carrying organisms there was a tendency to omit cell division at the normal cell length and this affect increased with increasing plasmid DNA content. It would be interesting to see if a similar increase in size, correlated with an increased number of plasmid markers existed for E. coli K12(TP120). Nordstrom et al.(1977) suggested that delayed cell division in plasmid-carrying strains was due to competition between plasmid and chromosomal DNA for some essential replication factor or to cross reactivation between repressors of replication. Plasmid DNA uses essentially the same replication apparatus as the bacterial chromosome (Goebel, 1973). Filaments are known to be induced by a number of factors that slow down or inhibit chromosome replication (Slater and Schaecter, 1974). It would be desirable to

examine chromosome elongation in E. coli K12 in the presence and absence of the plasmid TP120. Thus the increased cell volume of plasmid-carrying strains possibly associated with a reduced rate of chromosome replication could account for the growth differences demonstrated by evolved strains and their corresponding competitive ability. In conditions of lowest substrate concentration, that is at $D = 0.10 \text{ h}^{-1}$ in phosphorus and carbon-limited culture (Section 4), the competitive plasmid-minus strain exerts an even greater competitive effect than at higher dilution rates by reducing the uncompetitive organisms specific growth rate further. Results produced in this section have shown that there were greater differences in cell volumes and viable counts (Tables 5.2 and 5.3) at this lower dilution rate. Therefore it indicated that the small differences in viability of the organisms when growing separately are also important in mixed culture growth.

However, the affect of the presence of plasmid DNA on host cell growth, that is for viable counts and cell volume measurement, has not been widely investigated and it is not possible to extrapolate the situation found for the plasmid TP120 in E. coli K12 to other plasmids in different host organisms. For other organisms the degree of the affect may depend on the number of replication factors that the plasmid DNA has in common with the chromosomal DNA and the percentage of total DNA constituted

by the plasmid DNA. Also the small differences in growth shown between the organisms indicate that unknown additional factors, which have not been recognised here are also acting to increase the competitive ability of the evolved strain.

6. [¹⁴C] - GLUCOSE AND [³²P] - PHOSPHATE ASSIMILATION
IN THE PARENT STRAIN E. COLI K12(TP120) AND
EVOLVED STRAINS E. COLI K12(TP120A) AND E. COLI
K12(TP120E) (NO PLASMID).

The growth advantages demonstrated by evolved strains of E. coli carrying plasmids with fewer drug resistance markers compared to the parent strain were difficult to explain simply in terms of the conservation of energy sources in conditions of carbon and phosphorus limitation (Section 4). The results reported in Section 5 indicated that there were only small differences in the viability of the parent and evolved strains, in terms of biomass production and bacterial counts when the organisms were grown separately in phosphate-limited culture. Therefore the initial rates of phosphate and glucose uptake were measured for the organisms E. coli K12(TP120), E. coli K12(TP120A) and E. coli K12(TP120E) (no plasmid) in nutrient-limited continuous-flow culture. A more rapid nutrient uptake mechanism on behalf of the evolved strains might result in better growth and competitive advantages over the parent organism which would be manifested by higher specific growth rates. The organism with the more rapid uptake rate and subsequent metabolism would be capable of taking up and utilising any available

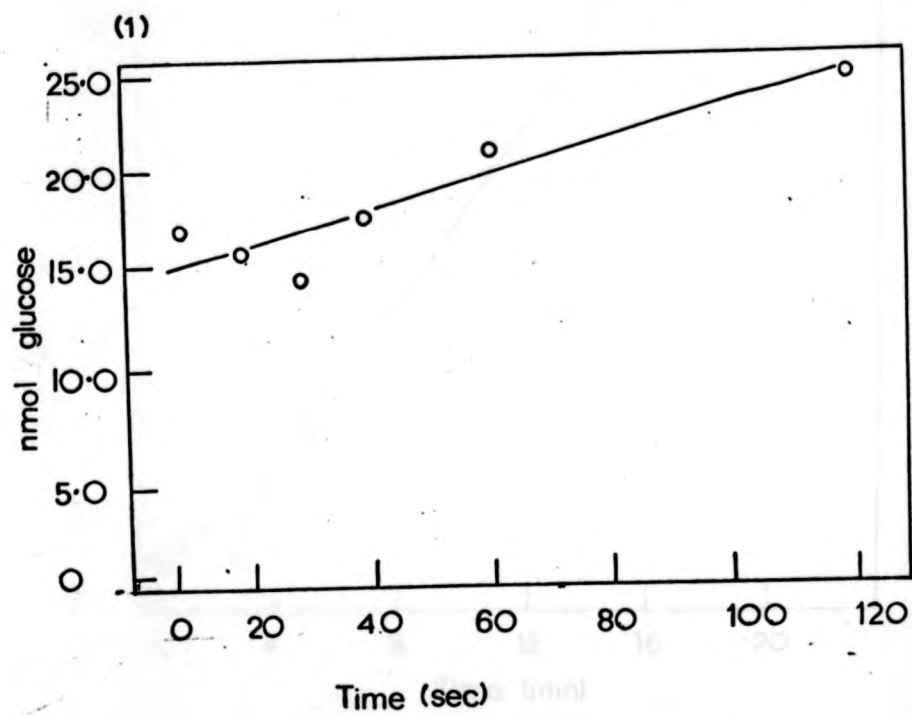
substrate more successfully than the slower growing organism. The initial or primary rate of assimilation was measured since during this phase the uptake rate is not likely to be complicated by subsequent metabolism of the substrate.

6.1. RATES OF $[^{14}\text{C}]$ - GLUCOSE ASSIMILATION AND CARBON DIOXIDE RELEASE IN E. COLI K12(TP120) and E. COLI K12(TP120A).

The initial rates of $[^{14}\text{C}]$ - glucose assimilation were measured. The rate of assimilation was determined for the parent organism E. coli K12(TP120) and the carbon-limited selection experiment evolved strain E. coli K12(TP120A) at high and low dilution rates. The strain E. coli K12(TP120E) (no plasmid) was not examined for the rate of glucose uptake. In mixed culture competition experiments the competitive strain had a greater growth advantage at the lower dilution rate of $D = 0.10 \text{ h}^{-1}$ and so any differences in rate of uptake may have been expected to be more apparent at low dilution rates (and low substrate concentrations.) Therefore steady state cultures at high and low dilution rates were harvested and uptake measured in batch cultures (Section 2.17) for high ($2.5 \mu\text{mol glucose ml}^{-1}$) and low ($0.5 \mu\text{mol glucose ml}^{-1}$) glucose concentration.

Figures 6.1 and 6.2 represent typical patterns of labelled glucose uptake shown by the different organisms.

Figure 6.1 Rate of $[^{14}\text{C}]$ - glucose assimilation measured with an initial glucose concentration $0.5 \mu\text{mol glucose ml}^{-1}$ for E. coli K12(TP120) grown at $D = 0.35 \text{ h}^{-1}$. (1) initial rate. (2) secondary rate.



(2)

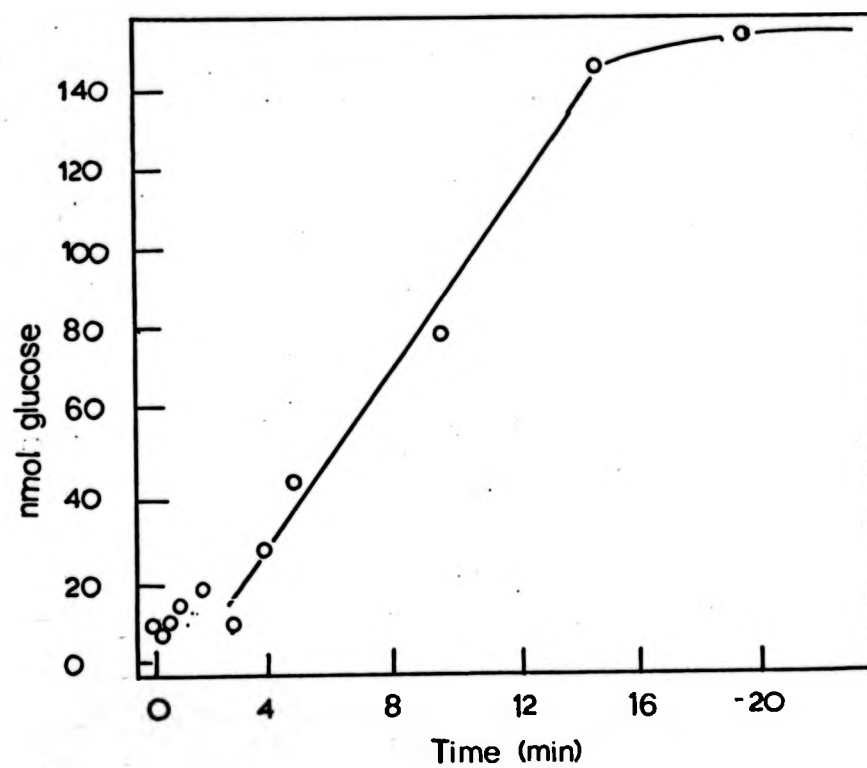
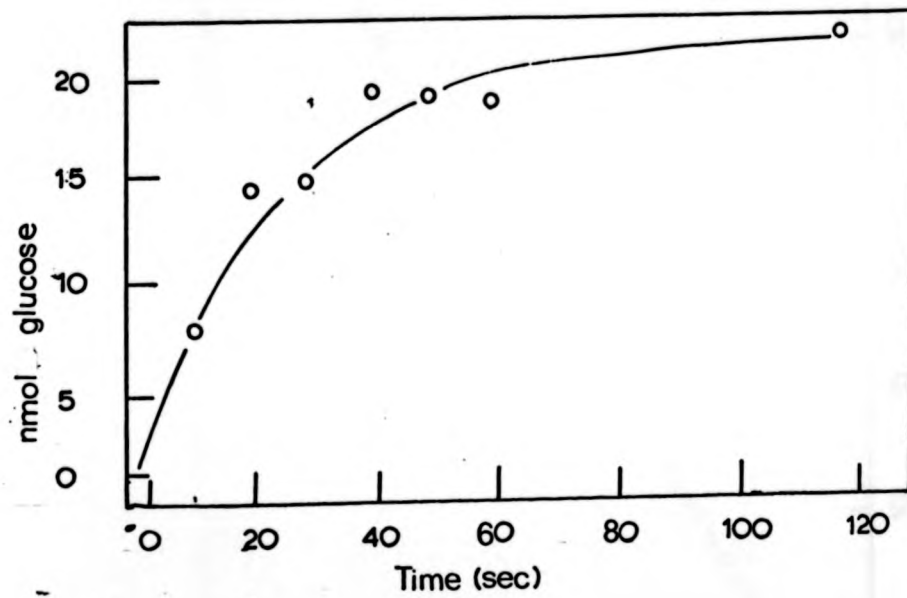


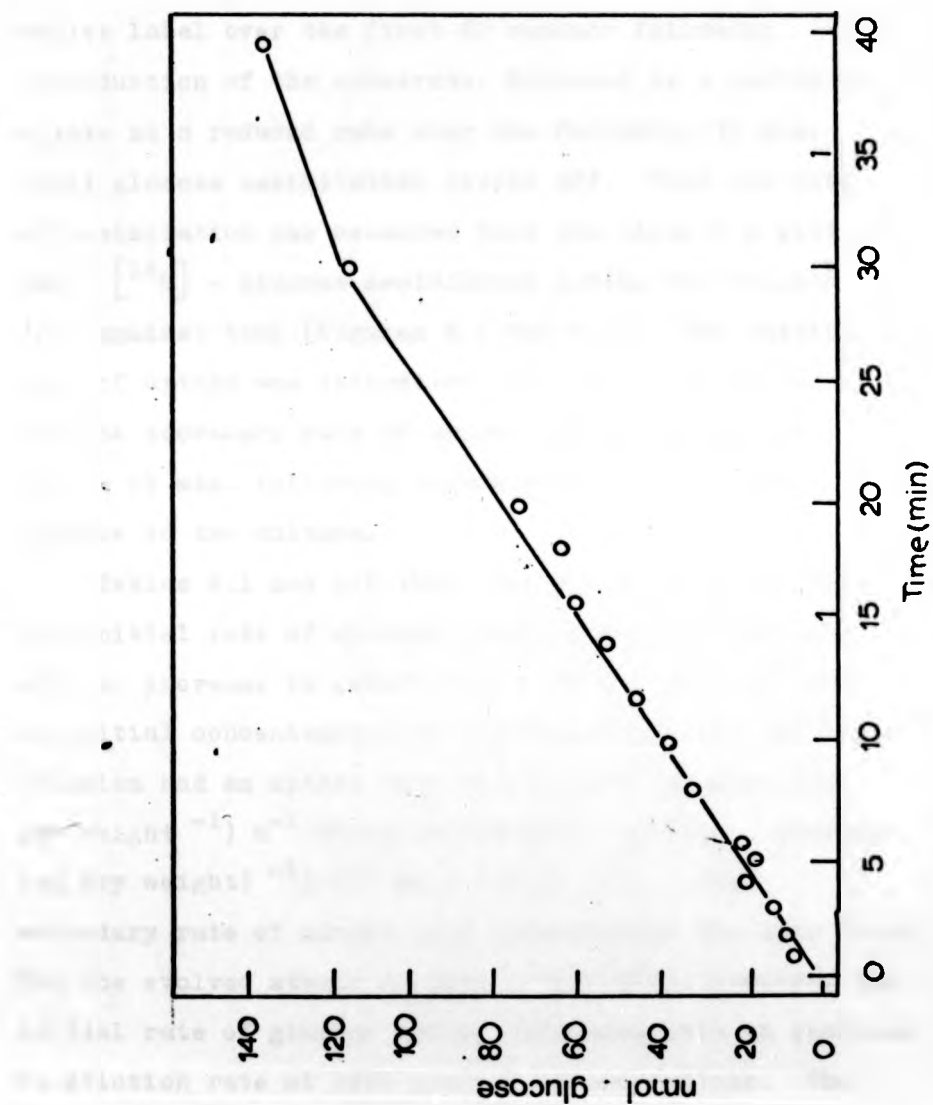
Figure 6.2. Rate of $[^{14}\text{C}]$ - glucose assimilation measured with an initial glucose concentration of $0.5 \mu\text{mol glucose ml}^{-1}$ for E. coli K12(TP120A) grown at $D = 0.35 \text{ h}^{-1}$. (1) initial rate. (2) secondary rate.

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This involved a rapid initial assimilation of radioactive label over the first 60 seconds following introduction of the substrate, followed by a period of uptake at a reduced rate over the following 30 min. until glucose assimilation levels off. Thus the rate of assimilation was measured from the slope of a plot of $\mu\text{mol } [^{14}\text{C}] - \text{glucose assimilated per (mg dry weight) h}^{-1}$ against time (Figures 6.1 and 6.2). The initial rate of uptake was determined over the first 90 sec. and the secondary rate of uptake during the period of 5 - 15 min. following introduction of labelled glucose to the culture.

Tables 6.1 and 6.2 show that for E. coli K12(TP120) the initial rate of glucose uptake increased slightly with an increase in growth rate. At $D = 0.10 \text{ h}^{-1}$ with an initial concentration of glucose of $2.5 \mu\text{mol ml}^{-1}$ the organism had an uptake rate of $120 \mu\text{mol glucose (mg dry weight}^{-1}) \text{ h}^{-1}$ which increased to $185 \mu\text{mol . glucose (mg dry weight}^{-1}) \text{ h}^{-1}$ at $D = 0.35 \text{ h}^{-1}$. The secondary rate of uptake also demonstrated the same trend. For the evolved strain E. coli K12(TP120A), however, the initial rate of glucose uptake decreased with an increase in dilution rate at both glucose concentrations. The secondary rate of glucose assimilation was similarly

Table 6.1 Rate of $[^{14}\text{C}]$ - glucose assimilation and $[^{14}\text{C}]$ - carbon dioxide release measured with an initial glucose concentration of $2.5 \mu\text{mol glucose ml}^{-1}$ for the parent (E. coli K12(TP120)) and the evolved strain (E. coli K12(TP120A)) at high and low dilution rates

Organism	Dilution rate (h^{-1})	Initial rate of $[^{14}\text{C}]$ - glucose uptake μmol^{-1} (mg dry weight) $^{-1}$ h^{-1}	Secondary rate of $[^{14}\text{C}]$ - glucose uptake μmol^{-1} (mg dry weight) $^{-1}$ h^{-1}	Rate of $[^{14}\text{C}]$ - CO_2 released (mg dry weight) $^{-1}$ h^{-1}
<u>E. coli</u> K12(TP120)				
	0.10	120.0	7.47	56.0
	0.35	185.0	12.33	50.0
<u>E. coli</u> K12(TP120A)				
	0.10	136.0	9.97	56.14
	0.35	130.0	8.96	55.38

Table 6.2 Rate of $[^{14}\text{C}]$ - glucose assimilation and $[^{14}\text{C}]$ - carbon dioxide release measured with an initial glucose concentration of $0.5 \mu\text{mol glucose ml}^{-1}$ for the parent (E. coli K12(TP120)) and an evolved strain (E. coli K12(TP120A)) at high and low dilution rates

Organism	Dilution rate (h^{-1})	Initial rate of $[^{14}\text{C}]$ - glucose uptake μmol^{-1} (mg dry wgt) h^{-1}	Secondary rate of $[^{14}\text{C}]$ - glucose uptake μmol^{-1} (mg dry wgt) h^{-1}	Rate of $[^{14}\text{C}]$ - CO_2 release μmol (mg dry wgt) h^{-1}
<u>E. coli</u> K12(TP120)	0.10	45.30	1.72	2.02
	0.35	58.99	6.35	2.81
<u>E. coli</u> K12(TP120A)	0.10	53.40	2.52	3.03
	0.35	29.94	2.50	4.77

reduced with increasing dilution rate such that for the initial glucose concentration of $2.5 \mu\text{mol glucose ml}^{-1}$ the secondary rate of uptake was reduced from $9.97 \mu\text{mol glucose (mg dry weight)}^{-1} \text{ h}^{-1}$ at $D = 0.10 \text{ h}^{-1}$ to $8.96 \mu\text{mol glucose (mg dry weight)}^{-1} \text{ h}^{-1}$ at $D = 0.35 \text{ h}^{-1}$.

Comparing the two organisms harvested at the same dilution rate and batch grown in the same glucose concentration, at the lower dilution rate the evolved strain had the more rapid rate of uptake. For the higher glucose concentration, E. coli K12(TP120A) was capable of a rate of glucose assimilation of $136.0 \mu\text{mol glucose (mg dry weight)}^{-1} \text{ h}^{-1}$ which was higher than the rate of $120 \mu\text{mol glucose (mg dry weight)}^{-1} \text{ h}^{-1}$ for the parent organism. With the initial glucose concentration, $0.5 \mu\text{moles ml}^{-1}$ the evolved strain had a rate of uptake 11% greater than that of E. coli K12 (TP120). However at the higher dilution rate of $D = 0.35 \text{ h}^{-1}$ the parent organism always demonstrated the more rapid assimilation rate (Figures 6.1 and 6.2) such that at the lower batch glucose concentration the parent strain had initial and secondary rates of uptake which were twice as rapid as the evolved strain (Tables 6.2).

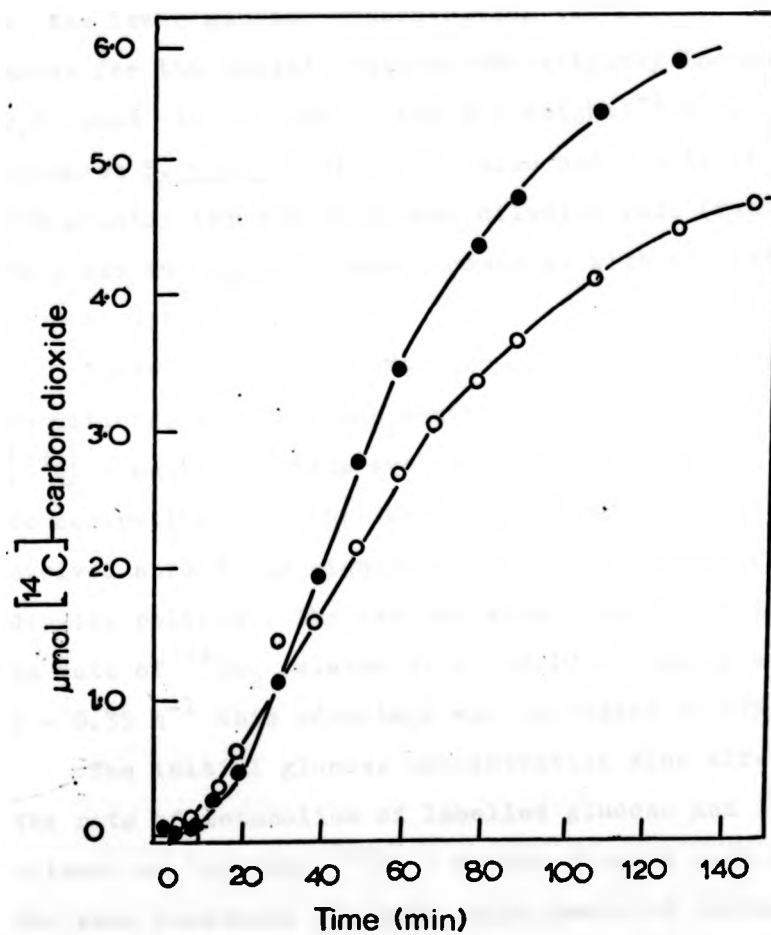
Overall the initial concentration of glucose appeared to influence the rate of uptake for both organisms

such that the initial and secondary rates of uptake were greater at the higher glucose concentration (Tables 6.1 and 6.2). The rate of uptake increased approximately 3 times for the increase in glucose concentration such that for $2.5 \mu\text{mol glucose ml}^{-1}$, E.coli K12(TP120) had an initial rate of uptake of $120 \mu\text{mol glucose (mg dry weight)}^{-1} \text{ h}^{-1}$ compared to only $45.3 \mu\text{mol glucose (mg dry weight)}^{-1} \text{ h}^{-1}$ at the concentration of $0.5 \mu\text{mol glucose ml}^{-1}$. The secondary rate of uptake was similarly influenced.

The rate of metabolism of glucose measured by the release of $^{14}\text{CO}_2$ was also determined in the same cultures in parallel experiments (Section 2.1.9). Figure 6.3 shows the typical pattern of release of labelled carbon dioxide over a period of 140 minutes for the parent and evolved strains. The release began slowly and then accelerated to an exponential rate of carbon dioxide evolution for the period starting approximately 20 minutes after inoculation of labelled substrate and ceasing at approximately 60 to 70 minutes.

For E. coli K12(TP120) the rate of labelled carbon dioxide release was reduced with an increase in dilution rate at the initial glucose concentration of $2.5 \mu\text{mol ml}^{-1}$ (Table 6.1). The organism E. coli K12(TP120A) showed approximately the same rate of $^{14}\text{CO}_2$ release

Figure 6.3. Rate of $[^{14}\text{C}]$ - carbon dioxide release measured with an initial glucose concentration of $2.5 \mu\text{mol glucose ml}^{-1}$ for E. coli K12(TP120) (\circ) and E. coli K12(TP120A), (\bullet) at $D = 0.35 \text{ h}^{-1}$.



(55 μmol) CO_2 (mg dry weight) $^{-1} \text{ h}^{-1}$) at both dilution rates for the higher glucose concentration (Table 6.1). At the lower glucose concentration the release of $^{14}\text{CO}_2$ shown for the parent organism was slightly increased from 2.02 μmol to 2.8 μmol (mg dry weight) $^{-1} \text{ h}^{-1}$. The organism E. coli K12(TPl20A) also had a rate of release 58% greater for the increased dilution rate (Table 6.2). This was an opposite result compared with the rate of glucose uptake.

Comparing the two organisms growing in the same conditions, both strains exhibited the same rate of $[^{14}\text{C}]$ - carbon dioxide evolution at the higher glucose concentration. At the lower concentration of glucose the evolved strain had significantly higher rates of carbon dioxide release. The evolved strain had a 33% increase in rate of $^{14}\text{CO}_2$ release at $D = 0.10 \text{ h}^{-1}$ while at $D = 0.35 \text{ h}^{-1}$ this advantage was increased to 67%.

The initial glucose concentration also affected the rate of metabolism of labelled glucose and its release as labelled $[^{14}\text{C}]$ - carbon dioxide such that the rate increased for both organisms with increasing glucose concentration.

Thus overall the only significant differences between the two strains in terms of rate of uptake and metabolism of labelled glucose were seen at the lowest substrate concentration.

- 1) At $0.5 \mu\text{mol glucose ml}^{-1}$ at $D = 0.35 \text{ h}^{-1}$ the parent organism had increased initial and secondary rates of glucose uptake compared to the evolved strain.
- 2) However, the evolved organism had a rate of $[^{14}\text{C}]$ - carbon dioxide release 67% greater than the parent strain.

Apart from these significant differences the two organisms exhibited very similar rates and trends of glucose uptake and metabolism such that:

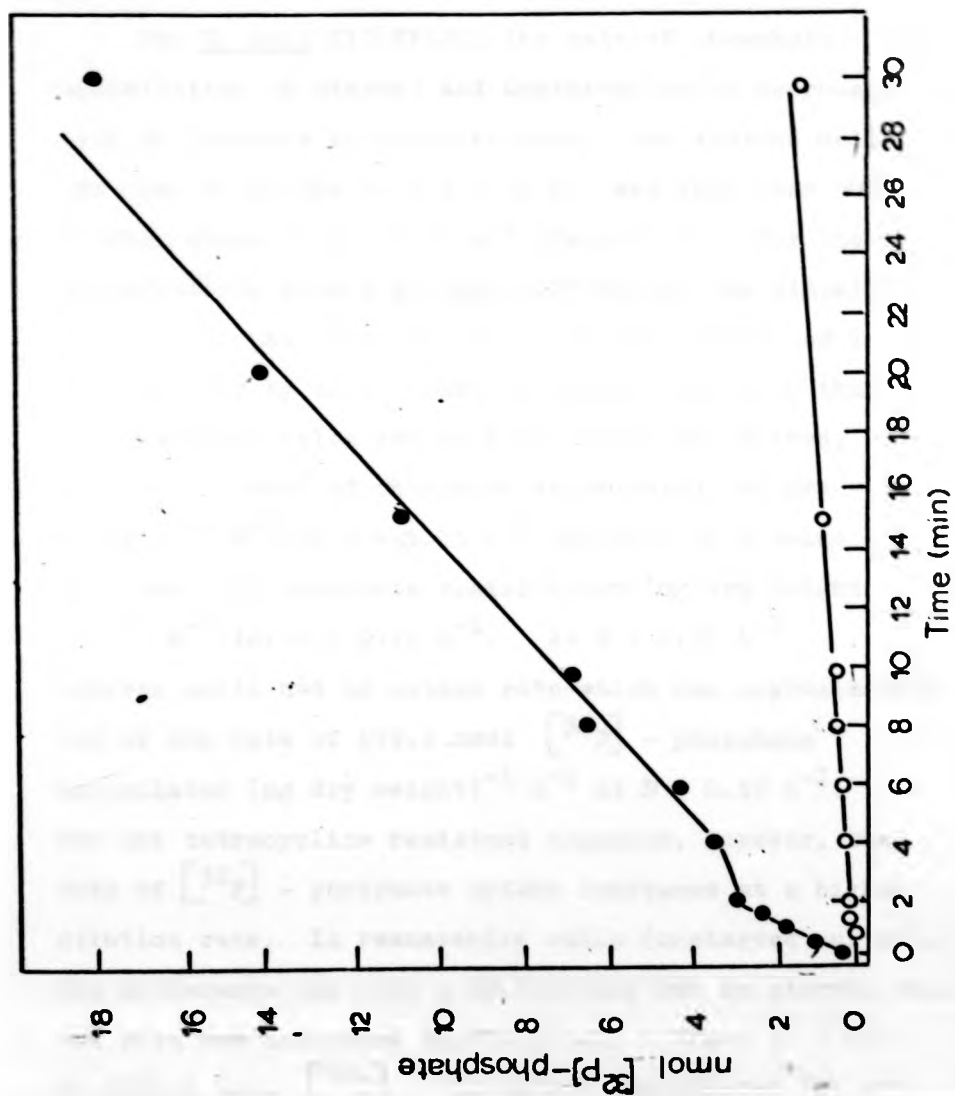
- 1) At the dilution rates of $D = 0.10 \text{ h}^{-1}$ and $D = 0.35 \text{ h}^{-1}$ at the highest glucose concentration the two organisms although differing in response to an increase in growth rate had very similar initial and secondary rates of uptake.
- 2) Again at the high glucose concentration the rate of metabolism and release of radioactive labelled carbon dioxide for the two organisms were very similar at a mean rate of $55 \mu\text{mol } ^{14}\text{CO}_2 (\text{mg dry weight})^{-1} \text{ h}^{-1}$.
- 3) The organisms had the same response to an increase in glucose concentration such that as the substrate concentration increased the rate of uptake and metabolism were simultaneously increased.

6.2 RATE OF $[^{32}\text{P}]$ - PHOSPHATE UPTAKE IN E. COLI
K12(TP120) AND EVOLVED STRAINS E. COLI K12(TP120A)
AND E. COLI K12(TP120E) (NO PLASMID).

The rate of $[^{32}\text{P}]$ - phosphate uptake was measured for the parent strain E. coli K12(TP120) the evolved strain E. coli K12(TP120A) and the plasmid-minus strain E. coli K12(TP120E) (no plasmid). The assimilation rate was determined for batch cultures harvested from steady state cultures growing at high and low dilution rates using the same dilution rates as in the mixed culture chemostat experiments (Section 4). Starved cells were incubated in phosphorus-free medium for a total of 3 h before the rate of $[^{32}\text{P}]$ - phosphate uptake was measured. The assimilation rate was determined at a batch phosphate concentration of $2.5 \mu\text{mol phosphate ml}^{-1}$.

The same pattern of phosphate uptake was seen for the parent and evolved strains. In unstarved cells the rate of phosphate uptake was at a constant rate over a period of 30 min. (Figures 6.4., 6.5 and 6.6). In organisms which had been starved for 3 h there was a biphasic pattern of uptake, beginning with a rapid, initial rate of $[^{32}\text{P}]$ - phosphate uptake for the first 2 min then continuing at a reduced rate until

Figure 6.4 Rate of $[^{32}\text{P}]$ - phosphate assimilation measured with an initial phosphate concentration of 2.5 $\mu\text{mol phosphate ml}^{-1}$ for E. coli K12(TP120) in starved (●) and unstarved (○) cell suspensions for organisms grown at $D = 0.10 \text{ h}^{-1}$.



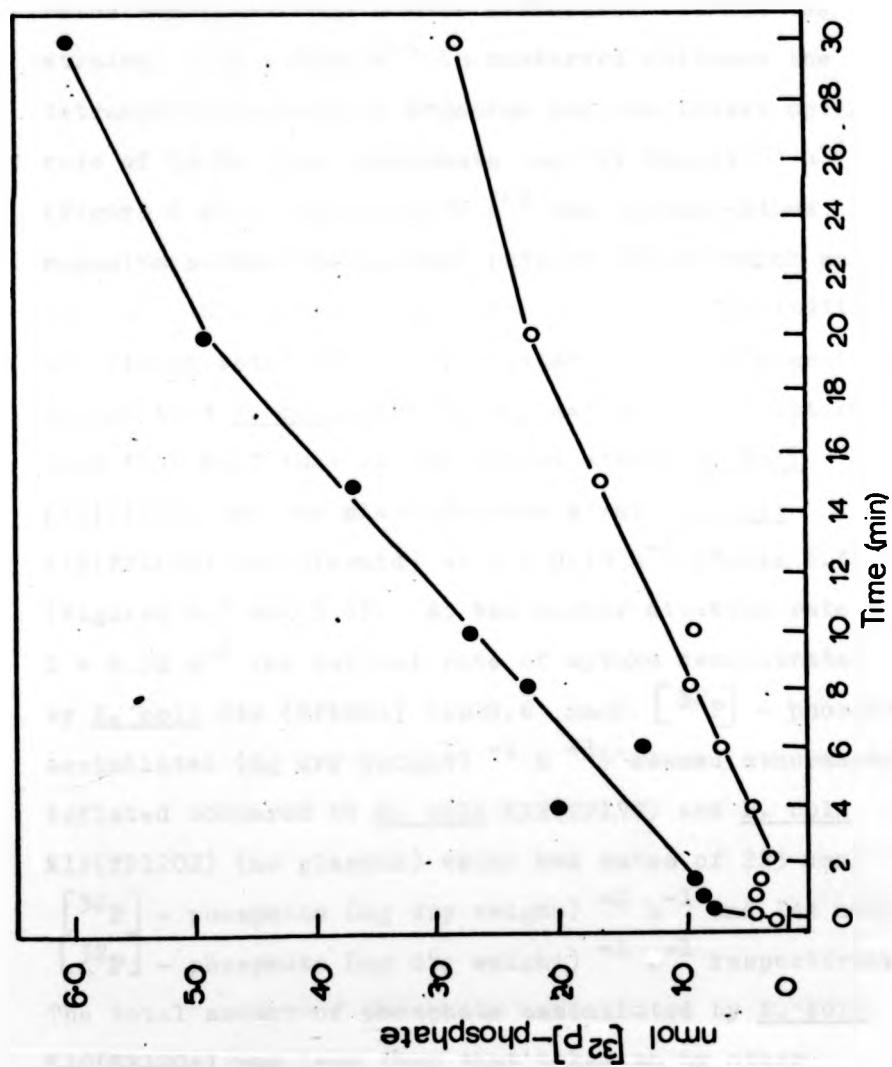
approximately 20 min. after introduction of $[^{32}\text{P}]$ -phosphate (secondary rate).

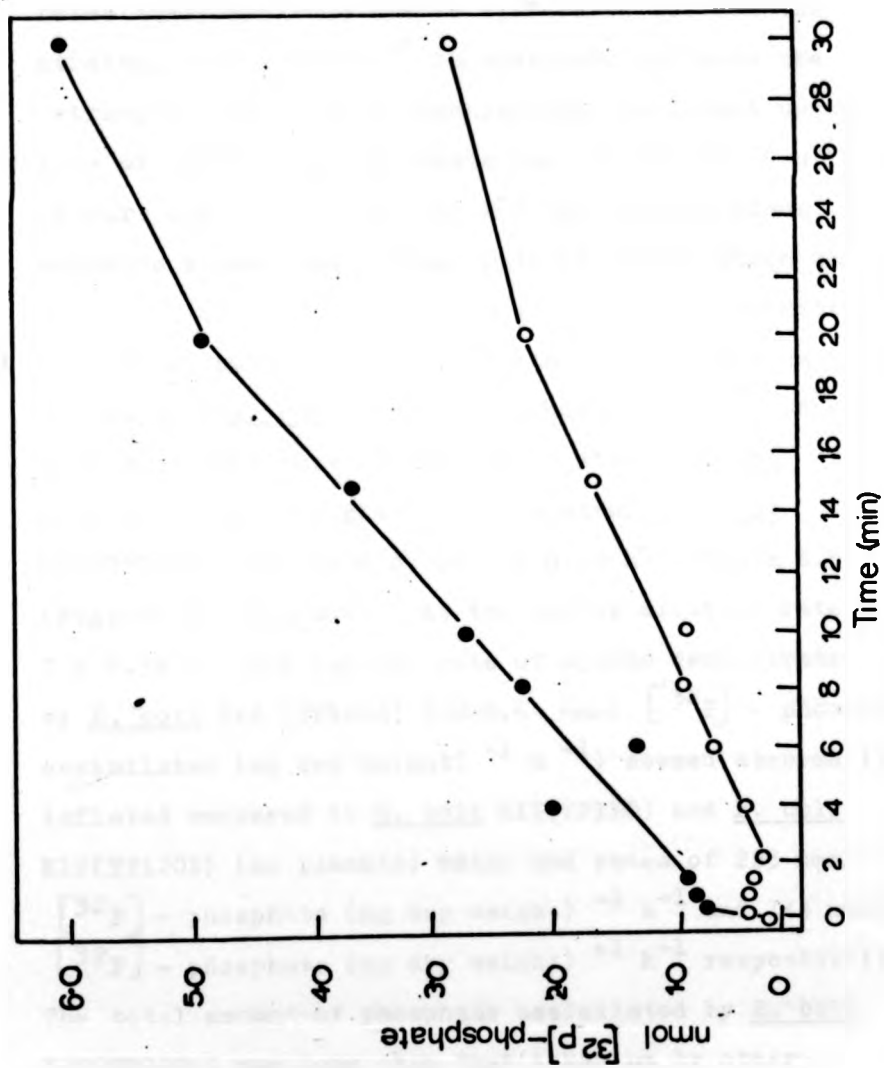
For E. coli K12(TP120) the rate of phosphate assimilation in starved and unstarved cells decreased with an increase in dilution rate. For starved cells the rate of uptake at $D = 0.35 \text{ h}^{-1}$ was less than 50% of that shown at $D = 0.10 \text{ h}^{-1}$ (Table 6.3). For the plasmid-minus strain E. coli K12(TP120E) (no plasmid) the rate of phosphate assimilation was influenced in the same way by an increase in growth rate such that for unstarved cells the rate of uptake was halved, that is 17.4 nmol of phosphate assimilated (mg dry weight) $^{-1} \text{ h}^{-1}$ at $D = 0.35 \text{ h}^{-1}$ compared to a value of 37.2 nmol of phosphate assimilation (mg dry weight) $^{-1} \text{ h}^{-1}$ for $D = 0.10 \text{ h}^{-1}$. At $D = 0.35 \text{ h}^{-1}$ starved cells had an uptake rate which was approximately 20% of the rate of 679.2 nmol $[^{32}\text{P}]$ - phosphate assimilated (mg dry weight) $^{-1} \text{ h}^{-1}$ at $D = 0.10 \text{ h}^{-1}$. For the tetracycline resistant organism, however, the rate of $[^{32}\text{P}]$ - phosphate uptake increased at a higher dilution rate. In resuspended cells (unstarved cultures) the difference was only a 5% increase but in starved cells the rate was increased approximately 5 times to a rate of 1029.6 nmol $[^{32}\text{P}]$ - phosphate assimilated (mg dry weight) $^{-1} \text{ h}^{-1}$ at $D = 0.35 \text{ h}^{-1}$ compared to a rate of only 236.4 nmol ^{32}P -phosphate assimilated (mg dry wgt) $^{-1} \text{ h}^{-1}$ at $D = 0.10 \text{ h}^{-1}$.

277

a

Figure 6.5. Rate of $[^{32}\text{P}]$ - phosphate assimilation measured with an initial phosphate concentration of $2.5 \mu\text{mol phosphate ml}^{-1}$ for E. coli K12(TP120E) (no plasmid) in starved (●) and unstarved(○) cell suspensions for organisms grown at $D = 0.10 \text{ h}^{-1}$.





Comparing the evolved strains and parent strain E. coli K12(TP120) when grown at the same dilution rates there was very little difference between the strains. A $D = 0.10 \text{ h}^{-1}$ in unstarved cultures the tetracycline-sensitive organism had the lowest uptake rate of $18.84 \text{ nmol phosphate (mg dry weight)}^{-1} \text{ h}^{-1}$ (Figure 6.4).. At $D = 0.35 \text{ h}^{-1}$ the plasmid-minus organism showed the slowest rate of uptake which was 24% less than that of the parent strain. Examination of primary rates of uptake in starved cell suspensions showed that E. coli K12(TP120A) had a rate of uptake less than half that of the parent strain E. coli K12(TP120) and the plasmid-minus strain E. coli K12(TP120E) (no plasmid) at $D = 0.10 \text{ h}^{-1}$ (Table 6.3) (Figures 6.5 and 6.6). At the higher dilution rate $D = 0.35 \text{ h}^{-1}$ the initial rate of uptake demonstrated by E. coli K12 (TP120A) ($1029.6 \text{ nmol } [^{32}\text{P}] - \text{phosphate assimilated (mg dry weight)}^{-1} \text{ h}^{-1}$) seemed abnormally inflated compared to E. coli K12(TP120) and E. coli K12(TP120E) (no plasmid) which had rates of $223 \text{ nmol } [^{32}\text{P}] - \text{phosphate (mg dry weight)}^{-1} \text{ h}^{-1}$ and $144 \text{ nmol } [^{32}\text{P}] - \text{phosphate (mg dry weight)}^{-1} \text{ h}^{-1}$ respectively. The total amount of phosphate assimilated by E. coli K12(TP120A) was less than that taken up by other organisms since the initial rate of uptake in E. coli K12(TP120A) occurred only for the first 60 sec. and

consequently may not have been a true measure of primary uptake rate.

As already described the starved and unstarved cell suspensions demonstrated different profiles of $[^{32}\text{P}]$ - phosphate assimilation. Starved organisms always had a rapid, initial rate of uptake (Figures 6.4, 6.5, and 6.6) followed by a secondary phase at a reduced uptake rate (Table 6.3) which was still greater than the rate of assimilation of unstarved organisms. This meant that E. coli K12(TP120) had an initial rate of uptake of $682.8 \text{ nmol } [^{32}\text{P}] \text{- phosphate assimilated (mg dry weight)}^{-1} \text{ h}^{-1}$ at $D = 0.10 \text{ h}^{-1}$ which was reduced to $30.4 \text{ nmol } [^{32}\text{P}] \text{- phosphate assimilated (mg dry weight)}^{-1} \text{ h}^{-1}$ during the second period whilst in unstarved cell suspensions the rate was only $27 \text{ nmol } [^{32}\text{P}] \text{- phosphate taken up (mg dry weight)}^{-1} \text{ h}^{-1}$. Different total amounts of $[^{32}\text{P}]$ - phosphate was accumulated in starved and unstarved cells. Starved cell suspensions assimilated 4 to 5 times the amount of $[^{32}\text{P}]$ - phosphate taken up by unstarved cell suspensions (Table 6.3). In starved cultures the amount of $[^{32}\text{P}]$ - phosphate taken up over the first 2 min., that is, during the primary phase, varied from 5 - 264 nmol of phosphate per 1×10^9 cells. In general the amount of phosphate assimilated

Figure 6.6. Rate of $[^{32}\text{P}]$ - phosphate assimilation measured with an initial phosphate concentration of $2.5 \mu\text{mol phosphate ml}^{-1}$ for *E. coli* K12(TP120A) in starved (●) and unstarved (○) cell suspensions for organisms grown at $D = 0.10 \text{ h}^{-1}$.



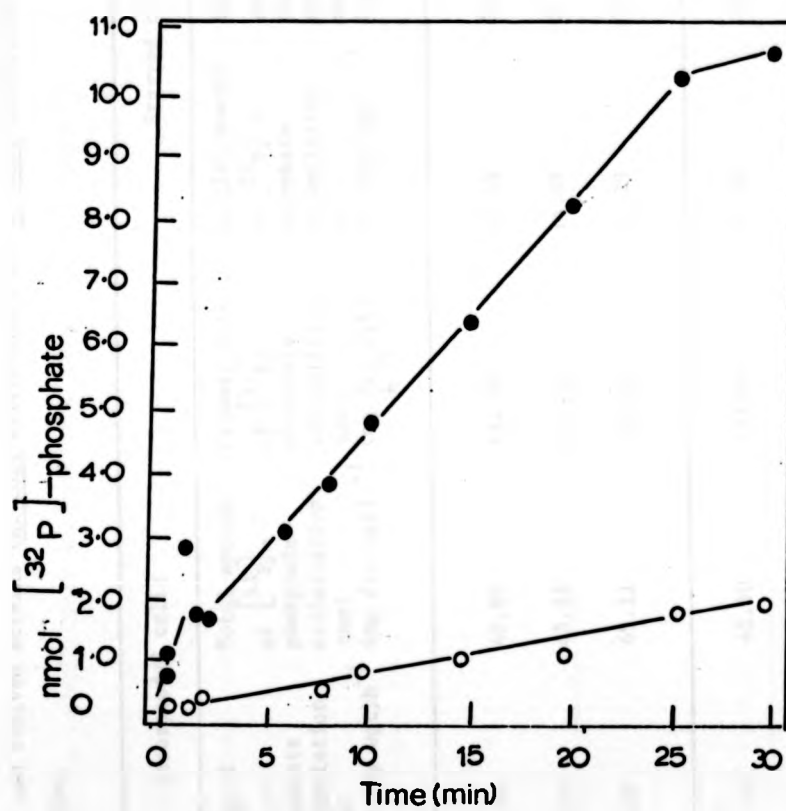


Table 6.3 Rate of $[^{32}\text{P}]$ -phosphate assimilation measured with an initial phosphate concentration of $2.5 \mu\text{mol. ml}^{-1}$ for the parent and evolved strains (*E. coli* K12(TP120A)) and *E. coli* K12(TP120E) (no plasmid) at high and low dilution.

Organism	Dilution rate	Resuspended cells			Starved cells		
		Rate of $[^{32}\text{P}]$ -phosphate assimilation $\text{nmol. (mg dry wgt)}^{-1}\text{h}^{-1}$	Total amount of $[^{32}\text{P}]$ -phosphate assimilation $\text{nmol. (mg dry wgt)}^{-1}$	Primary rate of $[^{32}\text{P}]$ -phosphate assimilation $\text{nmol. (mg dry wgt)}^{-1}\text{h}^{-1}$	Initial amount of $[^{32}\text{P}]$ -phosphate assimilation $\text{nmol. (mg dry wgt)}^{-1}$	Secondary rate of $[^{32}\text{P}]$ -phosphate assimilation $\text{nmol. (mg dry wgt)}^{-1}\text{h}^{-1}$	Total amount of $[^{32}\text{P}]$ -phosphate $\text{nmol. (mg dry wgt)}^{-1}$
<i>E. coli</i> K12(TP120)	0.10	27.00	40.80	682.80	13.50	302.40	183.00
<i>E. coli</i> K12(TP120A)	0.10	18.84	45.36	236.40	14.89	21.96	168.86
<i>E. coli</i> K12(TP120E)	0.10	37.20	69.11	679.20	5.29	73.80	156.80
<i>E. coli</i> K12(TP120)	0.35	21.60	41.30	223.20	89.70	188.46	243.00
<i>E. coli</i> K12(TP120A)	0.35	19.74	62.72	1029.60	50.65	134.40	285.00
<i>E. coli</i> K12(TP120E)	0.35	17.40	41.80	144.00	91.40	119.40	218.00

Table 6.4 Total amount of $[^{32}\text{P}]$ - phosphate assimilated during primary period of uptake in starved cell suspensions of the parent strain and evolved strains (E. coli K12(TP120A)) and E. coli K12(TP120E) (no plasmid) with an initial phosphate concentration of $2.5 \mu\text{mol ml}^{-1}$ at high and low dilution rates.

Organism <u>E. coli</u> K12	Dilution rate (h^{-1})	Phosphate assimilated nmol (1×10^9 cells) $^{-1}$
TP120	0.10	11.61
TP120A	0.10	9.95
TP120E	0.10	5.36
TP120	0.35	8.47
TP120A	0.35	19.13
TP120E	0.35	26.26

increased fractionally with increased growth rate (Tables 6.3 and 6.4).

In summary, there was little change in the rate of phosphate uptake for the evolved strains which were capable of rapid specific growth rates when compared with the parent organism E. coli K12(TP120) since:

- 1) All organisms showed the same phases of uptake typical of starved and unstarved cultures.
- 2) The rates of uptake for starved and unstarved cultures of the plasmid-carrying and plasmid-minus strains were similar, whilst the tetracycline-sensitive strain E. coli K12(TP120A) produced anomalous results for starved cell suspensions.
- 3) The total amount of $[^{32}\text{P}]$ -phosphate assimilated by the strains was uniform (Table 6.3).

6.3. DISCUSSION

The results reported in this section suggested that the rapid specific growth rate of evolved strains compared to the parent strain E. coli K12(TP120) (Section 4) was not due to an increased rate of assimilation for either carbon or phosphor^u, s in evolved strains. The evidence can be summarised as follows:

- 1) In $[^{14}\text{C}]$ - glucose assimilation experiments the

tetracycline-sensitive strain E. coli K12(TP120A) and the parent strain had almost equal initial rates of uptake such that at the higher dilution rate the parent strain even had the advantage.

2) The rate of $[^{14}\text{C}]$ - carbon dioxide release for the parent and evolved strains were also similar at the higher glucose concentration. At the reduced glucose concentration the rate of $[^{14}\text{C}]$ - CO_2 evolution was marginally higher for the evolved strain.

3) Both strains demonstrated increased rates of uptake with an increase in glucose concentration.

Neijssel, Hueting and Tempest (1977) have argued that glucose transport was not the growth limiting step except in glucose-limited cultures growing close to their maximum specific growth rates. Under the conditions examined this could only apply to E. coli K12(TP120) growing at $D = 0.35 \text{ h}^{-1}$ which was growing at 87% of its μ_{max} value. Thus it would be interesting to investigate the uptake of the evolved strains grown at dilution rates nearer to their μ_{max} values.

In the case of the $[^{32}\text{P}]$ - phosphate assimilation experiments the results may be summarised as follows:

1) There was little difference in the rates of uptake demonstrated by the three strains for starved cell suspensions. At $D = 0.10 \text{ h}^{-1}$ the tetracycline-sensitive

strain E. coli K12(TP120A) had a lower rate of uptake than the parent strain.

2) In organisms starved of phosphate the parent organism E. coli K12(TP120) showed an increased rate of uptake compared to the evolved strains. This suggested that the parent organism would be more capable of taking advantage of a sudden increase in phosphate concentration.

The rapid primary rate of uptake in starved cell suspension is likely to be due to the filling of a depleted phosphate pool. Medveckzy and Rosenberg (1971) described the same pattern of uptake in starved and unstarved cultures of E. coli K12 and quoted a phosphate pool size of 10 - 12 nmol phosphate per 1×10^9 cells. They suggested that the rapid uptake was achieved by high affinity and low affinity phosphate transport systems functioning simultaneously. In the organisms examined in this study the concentration of phosphate assimilated during this initial phase varied between 5 - 20 nmol phosphate per 1×10^9 cells (Table 6.4) and was higher in organisms grown at the higher rate. At the lower dilution rate of $D = 0.10 \text{ h}^{-1}$ E. coli K12(TP120) assimilated the highest phosphate concentration whilst at the higher dilution rate the plasmid-minus strain assimilated an amount 3 times that of the parent strain.

The amount and rate of phosphate uptake following the initial phase depended on the rate of removal of phosphate from the primary pool for esterification in the process of oxidative phosphorylation. In measurements of the secondary rate of uptake in starved cultures the plasmid-carrying strain had the fastest rate of uptake compared to the evolved strain. It is difficult to attribute this increase to an extra phosphate requirement in the plasmid-carrying strain since the differences in plasmid DNA content between parent and evolved strains is very small (Section 7.3).

Overall it appeared that the success of evolved strains in mixed culture chemostat competitive experiments was not due to the evolution of increased rates of uptake during selection experiments. This tended to disprove the argument that strains evolved in carbon or phosphorus-limited continuous-flow culture (Section 3) were selected not for a reduction in the cell's plasmid DNA content but as a result of the acquisition of a more efficient mechanism for phosphorous or carbon uptake. In mixed culture chemostat experiments the evolved strains were more competitive at the lower dilution rate. Uptake experiments did not show a similar advantage for the evolved strains at $D = 0.10 \text{ h}^{-1}$, for $[^{14}\text{C}]$ - glucose uptake the evolved strain did show a slight advantage at the lower substrate concentration.

Criticism could be made of the techniques used in measurement of nutrient uptake. For $[^{14}\text{C}]$ - glucose assimilation measurement, Herbert and Kornberg (1976) pointed out the limitations of the filtration procedure since they reported that for each mole of labelled glucose removed from the medium only 0.5 - 0.1 moles of glucose was measured in the filtered cells. This was because the remainder had been rapidly lost by oxidation to carbon dioxide and water. They also criticised the use of ice cold buffer for washing the filtered cells since this procedure led to loss of material through cold shock. Also they calculated from conversion rates of glucose to glucose-6-phosphate that the washing procedure would not have slowed down metabolism since the conversion was complete at 0.15 - 0.30 sec. following label uptake. Therefore it may have been an advantage to carry out the experiment at 4°C in order to examine the uptake in more detail. However this would not reflect the conditions found in the evolution or mixed population experiments which were carried out at 37°C . Moreover it was the difference in rates of uptake between parent and evolved strains which was the important factor and not the absolute rate of assimilation. Therefore since the measurement of relative strains took place under standardised experimental conditions the rates should be comparable.

7. ISOLATION AND MEASUREMENT OF PLASMID DNA

The parent strain E. coli K12(TP120) and the strains which had been evolved in either carbon or phosphorus - limited selection experiments (Section 3) and had consequently lost resistance to one or more antibiotics, were examined for the continued presence of plasmid DNA. This investigation was necessary since evolved strains may have lost the plasmid and concomitantly transferred their antibiotic resistance markers to a chromosomal position. If however the resistance markers remained on a plasmid then it was important to measure the plasmid size and examine any loss or insertion of DNA or other changes in the plasmid.

Two different techniques were used to confirm the presence or absence of extrachromosomal DNA within these strains (Sections 2.12.1 and 2.12.2). Pure plasmid DNA was isolated using dye-buoyant ethidium bromide - caesium chloride density gradient centrifugation while preparation of crude plasmid DNA was achieved by SDS lysis followed by phenol extraction and ethanol precipitation. The sizes of the plasmids were measured using the techniques of electron microscope contour length measurement and by agarose electrophoresis.

7.1. ISOLATION OF PLASMID DNA FROM THE PARENT STRAIN E. COLI K12(TP120) AND EVOLVED STRAINS.

The parent strain E. coli K12(TP120) and evolved strains E. coli K12(TP120A), E. coli K12(TP120B), E. coli K12(TP120C), E. coli K12(TP120D) and E. coli K12(TP120E) (no plasmid) were examined for the presence of plasmid DNA. Ethidium bromide - caesium chloride centrifugation (Section 2.12.1.) resulted in the separation of plasmid DNA from chromosomal DNA (Figure 2.3). Plasmid DNA was visualised in short wave ultraviolet light and appeared as a brightly fluorescing narrow band below a wider chromosomal DNA band. Using this technique plasmid DNA was isolated from all strains examined except the antibiotic sensitive strain E. coli K12(TP120E) (no plasmid) (Table 7.1.).

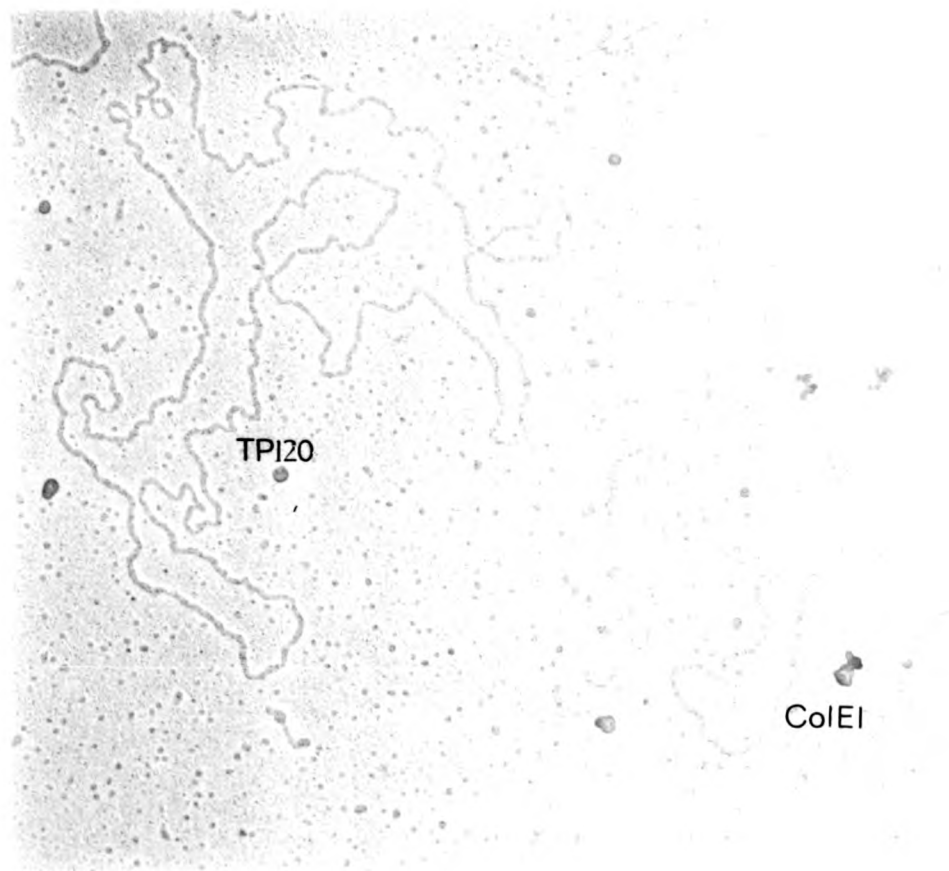
The strains were also examined using a crude lysate preparation followed by agarose electrophoresis. (Section 2.12.2). Plasmid DNA was revealed as brightly fluorescing narrow bands in agarose gels stained with ethidium bromide and illuminated by short wave UV illumination (Figure 7.2). Plasmid DNA was discovered in strains E. coli K12(TP120) (Ap, Sm, Su, Tc), E. coli K12(TP120A) (Ap, Sm, Su), E. coli K12(TP120B) (Sm, Su, Tc), E. coli K12(TP120C) (Ap, Sm, Su), and E. coli K12(TP120D) (Sm, Su) but could not be isolated from the strain E. coli K12(TP120E) (no plasmid) which had been

isolated from a phosphate-limited selection experiment (Section 3.3) and had apparently lost all plasmid-borne antibiotic resistance markers due to the loss of the whole plasmid (Table 7.1.).

7.2 DETERMINATION OF THE MOLECULAR WEIGHT OF PLASMID DNA BY ELECTRON MICROSCOPE CONTOUR LENGTH MEASUREMENT.

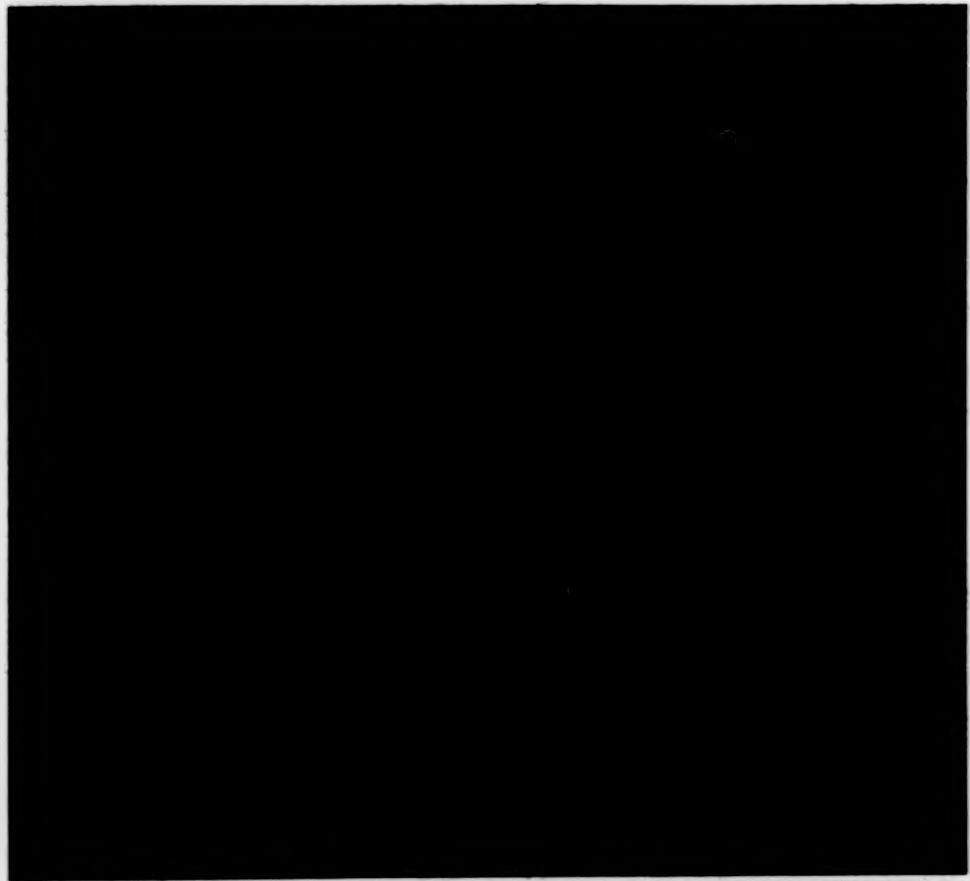
The length of double stranded plasmid DNA and its molecular weight was measured using the technique of Davis et al. (1971). The plasmid DNA was spread on a protein film and visualised by electron microscopy (Section 2.13.1). The small plasmid Cole1 - KH30 molecular weight 4.2×10^6 was used as an internal standard. Electron micrographs were taken of fields of view which contained the plasmid TP120 of unknown size, and the standard Cole1 - KH30 plasmid at 10,000 x to 20,000 x magnifications (Figure 7.1). Negatives were enlarged 10 to 50 times using a film projector and the plasmid DNA traced onto paper. The contour length was measured using a Jaker map measurer and subsequent calculations showed that the molecular weight of the plasmid DNA was 31.55×10^6 daltons ± 0.5 . This value agreed with the molecular weight of 31.7×10^6 calculated for this plasmid by Grindley et al. (1975).

Figure 7.1 (1) and (2) Electron microscope photographs of the plasmid TP120 including the smaller plasmid ColE1 used as the DNA molecular weight standard. (Stained with uranyl acetate, shadowed with platinum-palladium wire)



20000

(1)

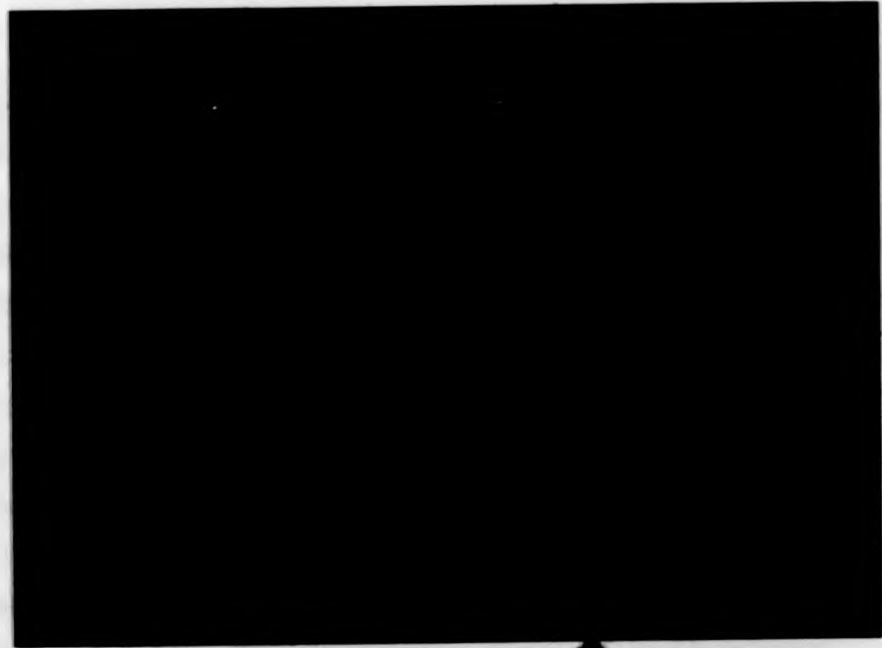


(x20,000)

7.1.1. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

7.1.2. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

(2)



(x10,000)

7.1.3. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

7.1.4. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

7.1.5. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

7.1.6. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

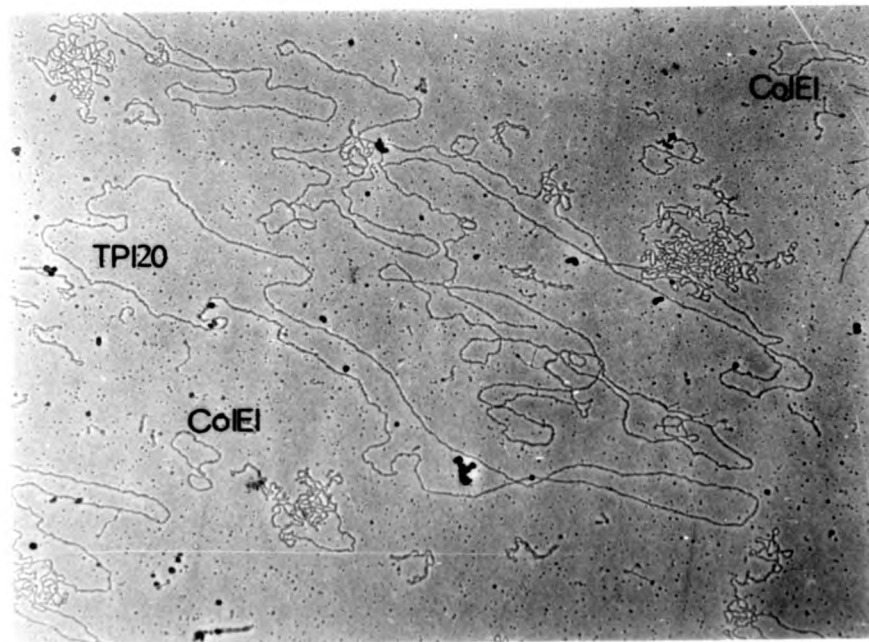
7.1.7. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

7.1.8. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

7.1.9. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

7.1.10. The following data, including a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, and the following, are the results of the following tests:

(2)



(x10,000)

This technique was, however, a lengthy and complicated process and consequently only the original plasmid TP120 was examined using this method.

7.3 DETERMINATION OF THE MOLECULAR WEIGHT OF PLASMID DNA OF PARENT AND EVOLVED STRAINS USING AGAROSE ELECTROPHORESIS

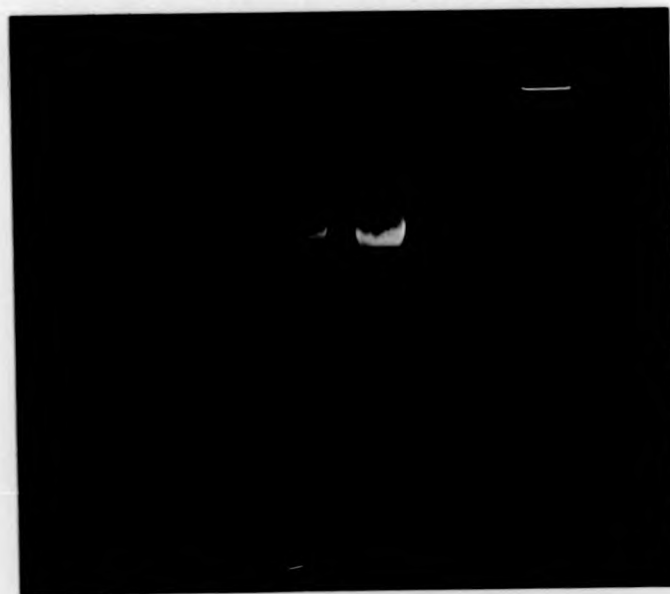
Since the measurement of plasmid DNA by contour length determination was a complicated procedure an alternative method was used. The technique of agarose gel electrophoresis has been extensively applied to the study of covalently closed circular (CCC) plasmid DNA (Meyer et al., 1976). This method was technically simpler but still allowed an accurate determination of plasmid size. Partially purified DNA obtained by ethanol precipitation of phenol-treated cleared lysates contained plasmid DNA which migrated on agarose gels usually as single bands at a rate inversely related to their molecular weight (Section 2.13.2. Figures 2.7, 7.2 and 7.3). The preparation also contained varying amounts of fragmented DNA, including chromosomal DNA, not removed in the preparation of cleared lysates of plasmid-carrying strains which consequently banded as a broad, diffuse area (Figure 7.2). By this technique the evolved strains E. coli K12(TP120A), E. coli K12(TP120B), E. coli K12(TP120C) and E. coli K12(TP120D)

were shown to carry plasmids which were smaller in size than the original plasmid TP120. Figure 7.2 shows the migration of the plasmid TP120 (Track 1), TP120A (Track 2), TP120B (Track 3), TP120C (Track 4) and plasmid TP120D (Track 5) in a 1% (w/v) agarose gel using a Tris-borate buffer at 120 V. Using plasmids R1 - 19 (molecular weight 62×10^6 daltons) pKM101 (molecular weight 23.7×10^6 daltons) and the plasmid cloning vector pACYC 184 (molecular weight 2.5×10^6 daltons) as standard DNA, the size of the original and evolved plasmids were calculated from a standard logarithmic plot of molecular weight against migration distance of plasmid DNA (Figure 7.3). The results summarised in Table 7.1 are made on the basis of 8 measurements of plasmid DNA on 0.80% (w/v) and 1% (w/v) agarose gels with Tris-acetate and Tris-borate buffer and with a combination of molecular weight markers. The molecular weight of the original plasmid TP120 measured by this technique agreed closely with the value of 31.55×10^6 daltons determined by contour length measurements. The evolved strains all contained plasmids that were smaller in size than the original plasmid. The plasmid TP120B which had lost resistance to ampicillin only and plasmid TP120D which had lost

Figure 7.2. Separation of plasmid and chromosomal DNA by agarose electrophoresis on a 1 % (w/v) gel using Tris-borate buffer pH 8.0. Partially purified preparations were subjected to electrophoresis for 3.5 h at 120V at room temperature. Track1, TP120; 2, TP120A; 3, TP120B; 4, TP120C; 5, TP120D; 6, pKM101; 7, R1 - 19; 8, pACYC 184.

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1 2 3 4 5 6 7 8



Plasmid DNA
Chromosomal
DNA
Plasmid DNA

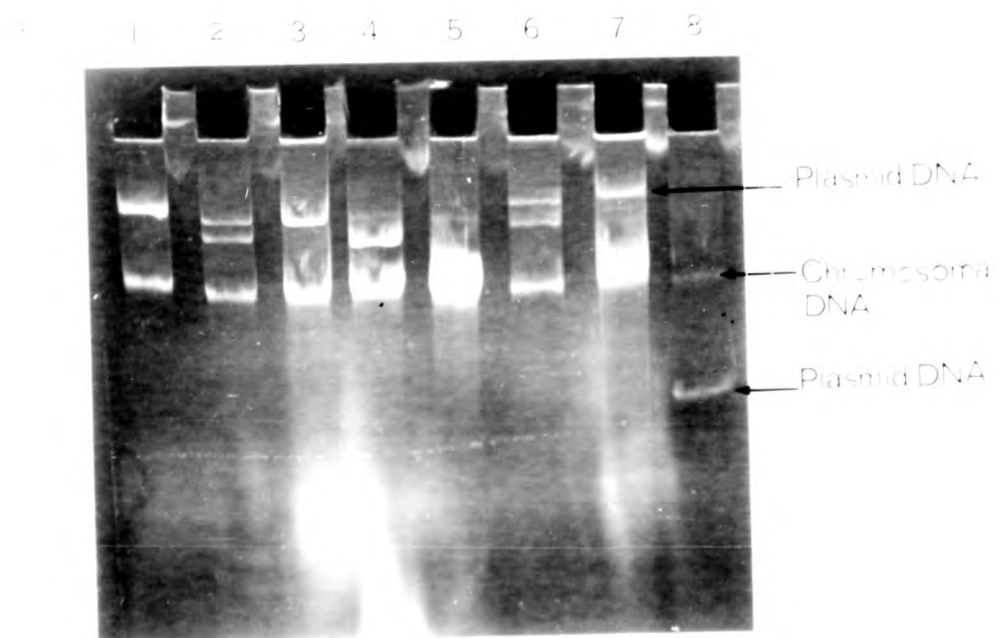


Figure 7.3. A plot of \log_e molecular weight against \log_e relative mobility for plasmid DNA shown in Figure 7.2. Where, \bullet = TPI20; \blacktriangle = TPI20B; \blacksquare = TPI20D; \square = TPI20A and \triangle = TPI20C.

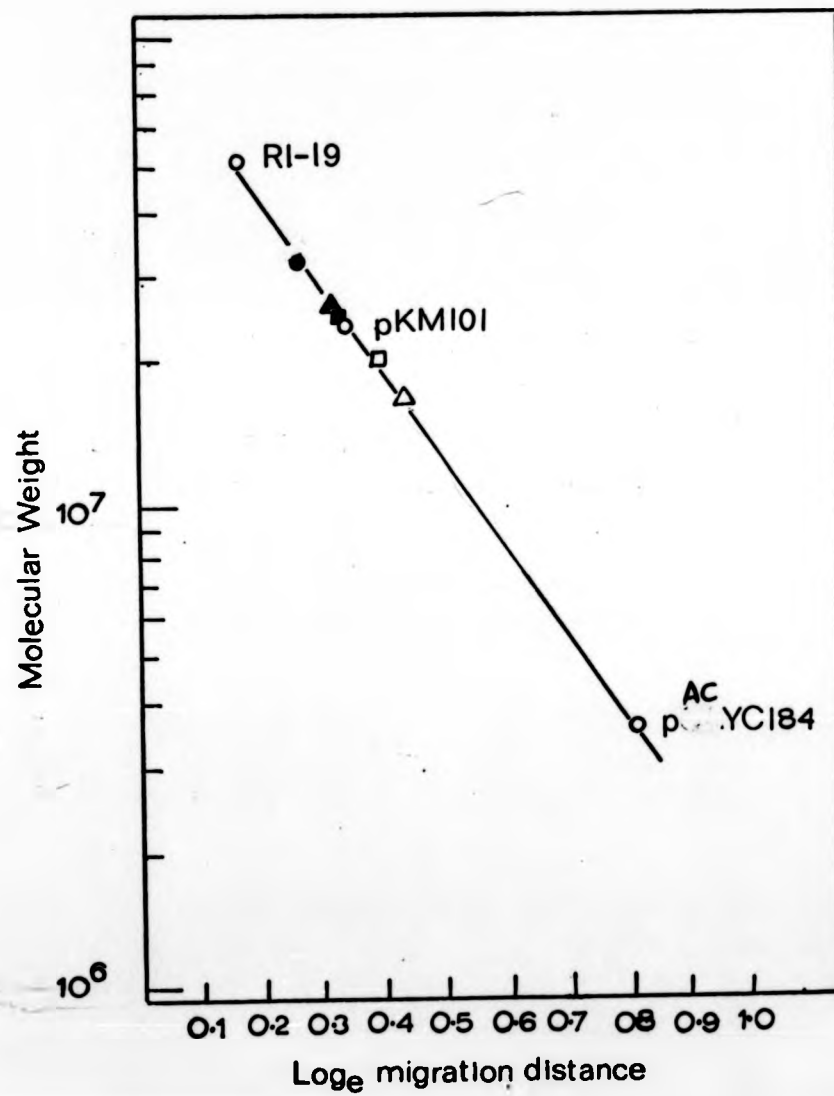


Table 7.1. The isolation and measurement of plasmid DNA from parent and evolved strains of E. coli K12

a. standard deviation calculated from measurement of 35 molecules.

b. standard deviation calculated from measurement on 8 x 0.8% and 1% agarose gels using Tris-acetate or Tris-borate electrophoresis buffer.

Host Strain (Plasmid markers)	Presence of Plasmid.		Molecular weight (x 10 ⁶) daltons		Size of fragment lost (x10 ⁶ daltons)
	CsCl centrifugation	Agarose electrophoresis	Electron microscope	Agarose electrophoresis	
<u>E. coli</u> K12(TP120) + (Ap, Sm, Su, Tc)	+		31.55 ±	31.6 ± 0	0
<u>E. coli</u> K12(TP120A) + (Ap, Sm, Su.)	+		ND	20 ± 0.4	11.6
<u>E. coli</u> K12(TP120B) + (Sm, Su, Tc.)	+		ND	24.9 ± 0.5	6.7
<u>E. coli</u> K12(TP120C) + (Ap, Sm, Su.)	+		ND	17.0 ± 0.45	14.7
<u>E. coli</u> K12(TP120D) + (Sm, Su)	+		ND	24 ± 0.5	7.6
<u>E. coli</u> K12(TP120E) - (no plasmid)	-		-	-	31.6

tetracycline resistance in addition were similar in size with molecular weights of 24.9×10^6 daltons and 24.0×10^6 daltons respectively. Surprisingly the plasmids TP120A and TP120C which had lost only tetracycline resistance were smaller than the plasmid TP120D which had lost two drug resistance markers including tetracycline. TP120C had a molecular weight of only 17.0×10^6 daltons which was 3×10^6 daltons smaller than TP120A. This loss was presumably implicated in the disappearance of transfer ability from this strain (Table 7.1).

The plasmid-carrying strains often showed two bands of plasmid DNA (Figure 7.2, Tracks 1 and 6). This is because open circular DNA generally migrates more slowly than the covalently closed circular form. (Aaij and Borst, 1972; Johnson and Grossman, 1977). Some of the covalently closed DNA must have been converted to this open circular form during the isolation procedure.

7.4 THE CHARACTERISATION OF PLASMID DNA BY ENDONUCLEASE DIGESTION

Strains evolved from nutrient-limited chemostat culture carried plasmids which were 6.7×10^6 - 14.7×10^6 daltons smaller than the parent plasmid TP120. Each plasmid carried a specific number of endonuclease digestion sites and consequently demonstrates a characteristic pattern of linear fragments of DNA of

different lengths during electrophoresis through an agarose gel. The loss of DNA from evolved plasmids may have involved the loss of DNA segments containing the endonuclease sites and, if this was the case, they should demonstrate a different digestion pattern when subjected to electrophoresis. The plasmids were therefore digested with the endonucleases EcoRI and BamHI (Section 2.14).

Figure 7.4 shows the agarose electrophoresis of the endonuclease digestion of the parent plasmid TP120 and evolved plasmids TP120A, TP120B, TP120C and TP120D by the enzyme EcoRI. Digestion of TP120 with EcoRI typically gives 4 bands of molecular weight of approximately 22.5×10^6 daltons, 4.4×10^6 daltons, 3.3×10^6 daltons and 1.2×10^6 daltons (J.W.Dale, personal communication). In Figure 7.4 the 3 larger bands are present but the smallest band of 1.2×10^6 daltons is absent probably due to the conditions used in the running of the gels. A higher agarose concentration would be required for demonstration of the smaller band but this would decrease the separation of the larger DNA bands. Digestion of the evolved strains resulted in the visualisation of a single wide band of linear DNA which contained linear DNA of around 22.5×10^6 daltons (Table 7.2). This band is

Table 7.2 EcoRI digestion of the plasmid TP120 and evolved plasmids:

Plasmid (markers)	Molecular weight of plasmids ($\times 10^6$ daltons)	Size of fragments ($\times 10^6$ daltons)
TP120 (Ap. Sm. Su. Tc).	31.55	22.5, 4.4, 3.3.
TP120A (Ap. Sm. Su)	20	22.0
TP120B (Sm. Su. Tc)	24.9	22.0
TP120C (Ap. Sm. Su. Trā)	17.0	22.0
TP120D (Sm. Su.)	24.0	22.0

Figure 7.4. Endonuclease digestion of the plasmid TP120 and evolved plasmids by the enzyme EcoRI. Electrophoresis was on a 1 % agarose gel using Tris-borate buffer pH 8.0 for 3.5 h at 120 V at room temperature. Track 1, TP120; 2, TP120A; 3, TP120B; 4, TP120C; 5, TP120D (partially digested).

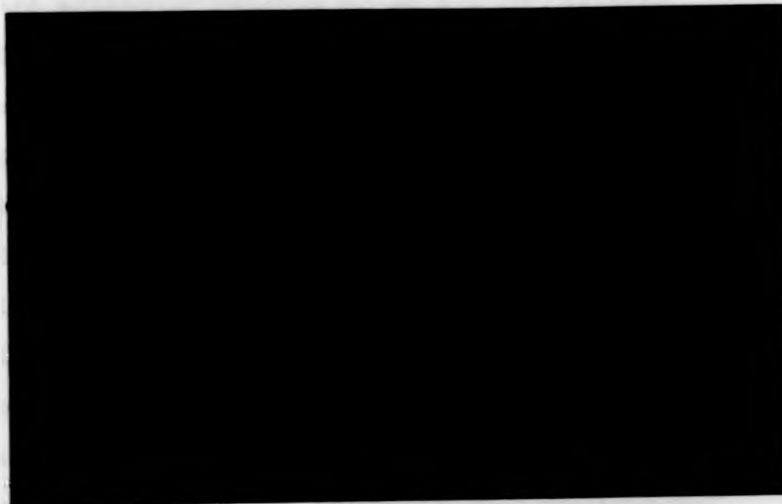
1

2

3

4

5



22.5×10^6
 4.4×10^6
 3.3×10^6

of a 10⁶ bp fragment (1.5 x 10⁶ bp) and a 10⁶ bp
 fragment (1.5 x 10⁶ bp). The fragment of 1.5 x 10⁶ bp
 of DNA was approximately 1.5 x 10⁶ bp. The fragment of 1.5 x 10⁶ bp
 and the 10⁶ bp fragment were approximately 1.5 x 10⁶ bp and 1.5 x 10⁶ bp
 and the smaller plasmid TPI20B (1.5 x 10⁶ bp) was only
 was only possible to visualize a single band, which
 indicated cleavage at only one site. The plasmid TPI20B,
 typically was only partially digested and as a result
 the linear fragment of DNA produced by cleavage at 1
 single BamHI site runs at the same level as the

plasmid

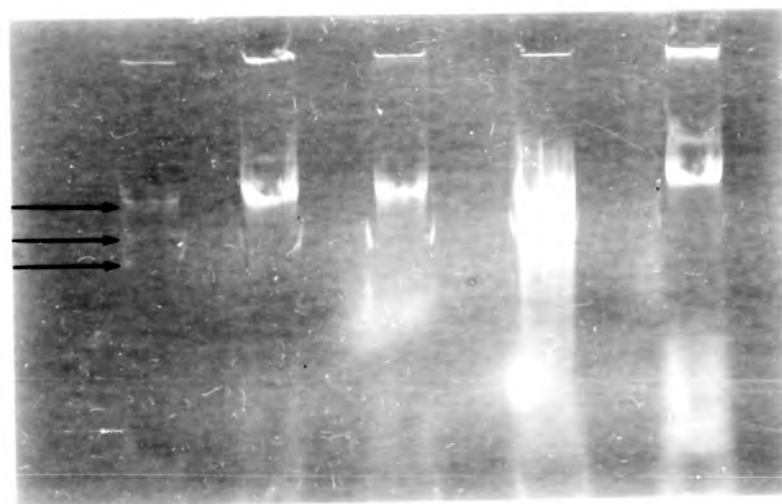
oRl.

ing Tris-

room

TPI20B;

200 x 10⁶
100 x 10⁶
50 x 10⁶



wide since it probably contains some undigested chromosomal DNA and fragmented plasmid and chromosomal DNA and so it is impossible to distinguish differences in the evolved plasmids. This indicated that the evolved strains had only a single site for the enzyme EcoRI. Since the plasmids TP120B and TP120D had molecular weights of 24.9×10^6 daltons and 24.0×10^6 daltons respectively it was expected that there should be an additional small DNA fragment, but this was not visualised. Therefore it appears that the two other sites of EcoRI digestion were situated on the segment of DNA which carried the ampicillin and tetracycline resistance markers.

Figure 7.5 and Table 7.3 show the result of digestion of the parent and evolved plasmid DNA with the endonuclease BamHI. The plasmid TP120 was cleaved in three places giving DNA fragments of approximately 22×10^6 daltons; 5.8×10^6 daltons; and 4.1×10^6 daltons (Table 7.3). The evolved plasmids had all lost at least one endonuclease site. The plasmids TP120A and TP120B were apparently nicked at two sites whilst for the smaller plasmid TP120C (17×10^6 daltons) it was only possible to visualise a single band, which indicated cleavage at only one site. The plasmid TP120D, typically was only partially digested and as a result the linear fragment of DNA produced by cleavage at a single BamHI site runs at the same level as the

Table 7.3 BamHI digestion of the plasmid TP120 and evolved plasmids

Plasmid (markers)	Molecular weight of plasmid ($\times 10^6$ daltons)	Size of fragments ($\times 10^6$ daltons)
TP120 (Ap. Sm. Su. Tc)	31.55	22, 5.8, 4.1:
TP120A (Ap. Sm. Su)	20	19, 3.1
TP120B (Sm. Su. Tc.)	24.9	21, 3.1
TP120C (Ap. Sm. Su. Trā)	17.0	15.0
TP120D (Sm. Su.)	24.0	22.0

Figure 7.5. Endonuclease digestion of the plasmid TP120 and evolved plasmids by enzyme BamHI. Electrophoresis was on a 1 % agarose gel using Tris-borate buffer pH 8.0 for 3.5 h at 120 V at room temperature. Track 1, TPI20D,(partial digest); 2, TP120C; 3, TP120B; 4, TP120A; 5, TP120; 6,TP120D.

1 2 3 4 5 6


 220×10^6
 5.8×10^6
 4.1×10^6

plasmid

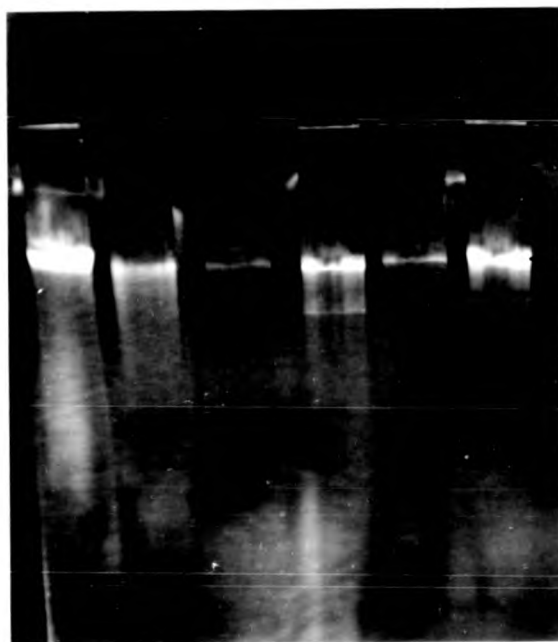
ing

at

digest);

, TP120D.

1 2 3 4 5 6



220×10^6
 5.8×10^6
 4.1×10^6

undigested, fragmented DNA and chromosomal DNA and produces a diffuse band, difficult to measure. Digestion of plasmid TP120A resulted in two bands of linear DNA which add up to a total of 22×10^6 daltons which is larger than indicated by measurement of undigested DNA in agarose electrophoresis (Table 7.1)

Since the evolved plasmids TP120A and TP120B had lost a single BamH1 site, compared to the parent strain and the remaining plasmids had lost two sites it appears that at least one of the BamH1 endonuclease sites is situated near to the EcoR1 sites which are apparently clustered in a segment of DNA carrying the drug resistance genes.

7.5 DISCUSSION

The results reported in this section confirmed that the loss of drug resistance markers from evolved strains was caused by an excision of DNA from the original parent plasmid. The smallest evolved plasmid was carried in the strain E. coli K12(TP120C) which had lost tetracycline resistance and transferability. Furthermore the drug sensitive strain E. coli K12(TP120E) (no plasmid) was shown to have lost the plasmid completely.

The DNA segments lost from E. coli K12(TP120A) and E. coli K12(TP120C) were 11.6×10^6 daltons and 14.7×10^6 daltons respectively. If the additional loss of DNA involved in the formation of E. coli K12(TP120C) was concerned with the loss of the transfer mechanism then the remaining segment of approximately 11.6×10^6 daltons presumably contained the tetracycline resistant determinant. This DNA fragment is much larger than a typical tetracycline resistant gene like the transposon Tn 10 which has a molecular weight of only 5.5×10^6 daltons (Table 1.2). Therefore the segment of DNA lost from the plasmid TP120 probably contained not only the tetracycline-resistance gene but also extra DNA coding for an unknown function. Similarly the loss of ampicillin resistance in the strain E. coli K12(TP120B) was caused by a loss of a segment of DNA 6.5×10^6 daltons in size from the parent plasmid TP120. Unlike most plasmids, TP120 (or R46) does not code for a TEM β -lactamase enzyme which is also responsible for ampicillin resistance in transposons Tn1 and Tn2 (Heffron, Sublett, Hedges, Jacob and Falkow 1976). Instead TP120 produces OXA β -lactamase which has not been reported as a transposon coded enzyme (Dale and Smith, 1974). The gene coding for the β -lactamase enzyme responsible for ampicillin resistance is thought

to be only $2.7 - 3.0 \times 10^6$ daltons which is less than 50% of the fragment of DNA lost from the parent plasmid during evolution of TP120B.

The plasmid pKM101, a transferable ampicillin resistant plasmid derived from the clinically isolated plasmid TP120 (or R46) by transduction with phage P22 followed by serial transfer has a molecular weight of only 23.7×10^6 daltons. This plasmid has lost resistance to tetracycline, streptomycin and sulphonamide by the excision of a length of DNA of approximately 8.0×10^6 daltons. The process which gave rise to pKM101 appears to be a more precise excision of DNA than that involved in the evolution of the plasmids TP120A, TP120B, TP120C and TP120D which have lost relatively larger amounts of DNA. It does however indicate that the drug resistance genes are clustered on a relatively small proportion of the plasmid DNA. Although it may be argued that the genes may have been inactivated by the loss of only small fragments of their total DNA. Further investigation by endonuclease digestion would be required to clarify this question. However the results do point to the conclusion that the loss of DNA from the parent plasmid is not the simple, precise excision of transposable elements. This may account for the relative stability of the parent plasmid particularly in carbon-limited conditions.

According to the definition of Cohen et al. (1978) all of the recognisable changes in the plasmid would be termed macroevolution. Macroevolution is thought to involve such processes as insertion, deletion or rearrangement of relatively large segments of DNA, that is, usually greater than 50 to 70 base pairs. All of the evolved plasmids have lost fragments of DNA of several thousands of base pairs. It is possible, however, that the plasmids also have undergone changes which Cohen et al. (1978) described as microevolution. This process is associated with the insertion, deletion or substitution of very short segments of DNA. Typically microevolution is detected by endonuclease digestion profiles. The endonuclease digestion studies of the plasmid TP120 reported in this section are rather unsatisfactory since the enzymes EcoRI and BamHI have few sites on the plasmid. It is really necessary to continue and improve the endonuclease digestion study using enzymes with a larger number of cleavage sites or using multiple digests with enzymes which have only a few sites. This relatively simple procedure should produce considerably more information about the structure of TP120 and evolution of TP120A, TP120B, TP120C and TP120D.

The procedures used in the measurement of plasmid DNA were more successful since the molecular weight determinations of the plasmid TP120 carried by the parent organism E. coli K12(TP120) agreed well for electron microscope and agarose electrophoresis techniques. This justified the use of the rapid agarose electrophoresis method alone for the measurement of evolved plasmids. Both methods have disadvantages and inaccuracies which can be minimised by the use of internal DNA standards. For electron microscope contour length measurements the length of the DNA molecule is known to depend on the conditions of mounting for the electron microscope. Davis et al. (1971) stated that it was a property of this method that the length of the DNA fluctuates around a mean value. The absolute length of the DNA decreases with ionic strength and DNA is further subject to uncontrollable variations from grid to grid even when the grids are mounted under seemingly identical conditions. This can be seen when comparing the appearance of the plasmid DNA shown in Figures 7.1.1 and 7.1.2 which had been prepared under exactly the same conditions. The relevant variables are concerned with film pressure and density and also the amount of cytochrome collapsed around the DNA (Davis et al. 1971). All these factors were reduced in effect by the

inclusion of ColE1 - KH30 as the internal standard.

For molecular weight measurements of plasmid DNA by agarose electrophoresis the interpretation of the plasmid bands can be confused by the presence of open and linear forms of DNA. Open and linear forms of DNA appeared infrequently in partially purified DNA preparations and usually produced fainter bands than the covalently closed circular forms. Willshaw et al. (1979) found that open circular molecules were not usually found in preparations of plasmids which were larger than 20×10^6 daltons and smaller than 60×10^6 daltons. They were thought to be caused by prolonged storage and repeated freezing and thawing. Freshly prepared samples of the partially purified DNA allowed accurate measurement of the plasmid DNA.

The evolved strains which were shown to carry smaller plasmids coding for fewer drug resistances had increased maximum specific growth rates compared to the original parent strain (Section 3.5) In Davis and Mingioli medium the μ_{\max} values appeared to increase with a decrease in plasmid size (Table 3.7) such that E. coli K12(TP120B) which contained a plasmid of molecular weight 24.9×10^6 daltons had a μ_{\max} of 0.46 h^{-1} compared to E. coli K12(TP120C) which had a plasmid of 17×10^6 daltons which had a μ_{\max} of 0.57 h^{-1} .

However, in Hershey defined medium (Table 3.8) the same relationship was not seen. In fact E. coli K12(TP120B) demonstrated a faster maximum specific growth rate than E. coli K12(TP120C). This confirms the proposition that whilst the decrease in plasmid size has influenced the specific growth rate there are other interacting factors. For example, although the strain E. coli K12(TP120D) carried a plasmid which is larger than the plasmids contained in E. coli K12(TP120A) and E. coli K12(TP120C) it codes for the expression of only two drug resistances and in Hershey defined medium this gives a growth advantage over E. coli K12(TP120A) and E. coli K12(TP120C) (Table 3.8). Therefore it is not only the amount of DNA replicated but also the expression of the DNA which influences the specific growth rate. Adams et al. (1979) found that the success of competing strains carrying different plasmids of different sizes depended not only on plasmid size but the functions expressed by the plasmid. Moreover it appears that some growth advantage has been gained by organisms selected from either phosphate or carbon-limited chemostat culture. This means that organisms which had evolved in phosphate-limited selection experiments (Section 3.3) had a growth advantage over

those strains which had been evolved in carbon-limited experiments when grown in Hershey defined medium (Section 3.5, Table 3.8). The reverse situation was also true and was confirmed in competition experiments described in Section 4.4. Therefore the strains isolated from a particular nutrient limitation had probably evolved a more efficient metabolism of that substrate although this was not obviously related to glucose or phosphate assimilation (Section 6).

This means that the increased μ_{\max} values demonstrated by the evolved strains were due to a complex combination of effects involving a reduction in DNA content and expression, with an independent more efficient carbon or phosphate utilisation.

8. DISCUSSION AND GENERAL REMARKS.

A number of interesting and significant observations have been made on the behaviour of the N group plasmid in cultures of E. coli K12(TP120) grown in an environment limited in phosphorus or carbon and lacking a drug selection pressure. In view of the strongly selective growth conditions occurring in chemostat continuous-flow culture, due to nutrient limitation and imposed high and low growth rates (Cox and Gibson, 1974; Harder et al., 1977) and also because of the large differences in specific growth rates of plasmid-carrying and plasmid-minus strains of E. coli K12 (Tables 3.6., 3.7 and 3.8) it was anticipated that evolved plasmid-minus strains would have been rapidly selected for under nutrient-limited conditions. However selection experiments (Sections 3.2. and 3.3) showed that this was not the case and indeed in only one instance, under phosphate-limited conditions after approximately 700 h growth was a plasmid-minus strain isolated. Thus the plasmid TP120 was considered to be surprisingly stable in apparently unfavourable growth conditions. In comparison with other plasmids, which have been subjected to a similar examination, it was considerably more stable than the F'_{lac} plasmid of E. coli grown in lactose-limited conditions (Slater and Bull, 1978) or the tetracycline-resistant plasmid grown in E. coli and described by Jones and Primrose, (1979). However it was apparently less stable than the RPl plasmid of E. coli strain W3110 which was stably maintained for up to 144 h growth in conditions of carbon, phosphorus or magnesium

limitations at dilution rates between 0.05 h^{-1} and 1.0 h^{-1} (Melling et al., 1977)

In the case of the plasmid TP120, rather than the loss of the whole plasmid, the plasmid more readily evolved to produce a smaller plasmid which lacked one or more drug resistance markers and which maintained a growth advantage over the parent organism. Bayley, Duggleby, Worsey, Williams, Hardy and Broda (1977) described the loss of the toluate degrading function from the Tol plasmid carried by P.putida mt-2 which was caused not by the disappearance of the whole plasmid (M.W. 78×10^6 daltons) but by the loss of a segment of only 27×10^6 daltons in size. This illustrated the point that whilst the majority of plasmids have been isolated due to drug resistance or metabolic coded functions, these properties may occupy only a fraction of the plasmid's genetic capability. Therefore the remaining segment of the plasmid could code for functions which although difficult to identify give a selective advantage to the organism (Adams et al., 1979). These functions may be very varied, Adams et al., (1979) has suggested that the presence of IS elements on the plasmids would bestow an advantage on the host organism because of the increased potential for recombination. In a review by Bennet and Richmond (1978) they suggested that extrachromosomal elements provided sites for possible gene duplication thereby increasing the genetic flexibility of the organism by permitting non-lethal mutations. In this study the UV resistance property coded for by the plasmid did not receive attention. Typical of certain R factors and Col factors, plasmid TP120 has been shown to

reduce the bactericidal effect of UV irradiation in Salmonella typhimurium hosts but in doing so increased its mutagenic effect as measured by a higher frequency of spontaneous reversion of certain mutations causing a change to auxotrophic character (Mortelmans and Stocker, 1976). The UV protection mechanism involving enhancement of UV mutagenesis is thought to reflect a plasmid determined ability to effect repair of UV damaged DNA by some error prone mechanism. Therefore this process is capable of protecting the host organism whilst providing a potential for the introduction of genetic variability into the population. The parent plasmid may similarly harbour other advantageous properties which are maintained in the smaller, evolved plasmids TP120A, TP120B and TP120D and therefore account for the low frequency of plasmid-minus organisms. Since the evolution of ampicillin and tetracycline sensitive organisms was achieved by the loss of relatively small segments of DNA which constitute only approximately 20% of the plasmid there is a substantial remaining portion of DNA whose function is unknown and could be coding for such advantageous properties.

Having demonstrated that the possession of the plasmid TP120 by E. coli K12 was disadvantageous to the organism when in competition with evolved strains which carried smaller plasmids, or even more so with plasmid-minus strains, E. coli K12(TP120) was still maintained for relatively long periods in carbon-limited and phosphate-limited conditions as a residual population (0.0001 - 0.1% of the total population) which was never totally lost from the population.

In vivo the examination of faecal samples from subjects who had not received antibiotic therapy in recent time showed that there was a high probability that the faecal flora contained a few resistant E. coli (Datta, 1969); a situation perhaps analagous to the residual population found in chemostat selection and competition studies. Similarly carbenicillin resistant P. aeruginosa strains which arose in a burns unit in a Birmingham hospital (Lowbury et al. 1969) in 1967, disappeared when the antibiotic was withdrawn from use but when it was re-introduced a year later resistant isolates began to appear once more (Sykes and Richmond, 1970). These strains carried the identical plasmid to that isolated in 1967 and points to the probability that, as in chemostat culture, the plasmid remained in a small residual population which was selected for when the antibiotic was re-introduced. Similar observations of residual resistant populations have been reported by Melling et al. (1977) and Adams et al. (1979) for chemostat populations involving the maintenance of an organism carrying a plasmid coding for apparently unnecessary information, as a small percentage of the total population. Similarly in competition experiments with obligate and facultative chemolithotrophic Thiobacillus species (Smith and Kelly, 1979) the uncompetitive strain was never completely removed from the chemostat as predicted theoretically. This provides a means of maintaining diversity within a population and reduces the need for the whole of a bacterial population to express a

particular character. If, for example, the resistance to antibiotics can be readily acquired from among a few members of resistant bacteria when challenged, the population is at a growth advantage when grown in a drug-free environment in conservation of energy and elemental resources. Therefore it would be interesting to examine the changes occurring in the chemostat population which was present at the termination of a typical selection experiment when challenged by a single drug. Would this result in the re-establishment of the parent population E. coli K12(TP120) or the transfer of the selected resistance marker from the residual population to one of the evolved strains which may still maintain a growth advantage?

Although the results reported for the stability of the plasmid TP120 appear to find analogies in the in vivo situation the limitation of the chemostat culture must be realised. Obviously the nutrient-limited conditions described in this investigation cannot completely reproduce the diverse situations occurring in natural environments where plasmid-carrying organisms survive and transfer their extrachromosomal elements. Plasmid-bearing bacteria have been isolated from the intestines of animals, soil, river water, sewage and from skin, blood and urine infections. There is a scarcity of published data describing which nutrient finally limits growth in natural situations. For example, enteric bacteria are assumed to exist in fresh water under starvation conditions with growth limited by the non

availability of a suitable carbon source (Hendrick, 1972). However the populations may be simultaneously limited by phosphorous or nitrogen which are only present at higher concentrations as a result of pollution (Brown, 1977). In contrast organisms growing in animal intestines exist in an anaerobic environment which is nutrient rich, but supports a dense microbial population. In blood infections nutrient limitation is severe but iron is normally considered to be the growth limiting nutrient (Sussman, 1974) although inorganic phosphate or even zinc (Weinburg, 1974) have also been implicated.

Moreover the growth rates achieved by populations growing in nature are usually very low. For example, in freshwater environments E.coli has been shown to grow at a specific growth rate as low as 0.003 h^{-1} which is far lower than the lowest dilution rate of $D = 0.10 \text{ h}^{-1}$ considered in this study. The advantage of evolved strains over the parent strain increased with the decrease in dilution rate so that in a natural environment the plasmid-carrying organism may be at a greater disadvantage. It would be desirable to examine plasmid stability in chemostat culture maintained at these reduced growth rate conditions.

Another limitation of the chemostat is that it cannot reflect the growth medium of the natural situations. The inefficient transfer of plasmid DNA shown by E. coli K12(TP120) is typical of N group plasmids and is considered to be associated with their predominance in soil growing organisms where cell to cell contact necessary for

conjugation is easily achieved (Dennison and Baumberg, 1975). Gut bacteria also have an abundance of surfaces and due to the density of the population an increased chance of cell to cell contact. In river water however nutrient limitation can be severe and organisms relatively fewer in numbers and they also may have fast flowing currents to contend with and cell to cell contact may be more difficult to achieve. The chemostat environment approximates most closely to this fresh-water situation where the growth media is aerated and stirred at such a rate that the transfer rate is apparently reduced to zero. In the case of the plasmid TPl20 transfer and retransfer of plasmid DNA did not appear to be an important factor in the maintenance of the plasmid within the population since the majority of organisms carried a copy of the plasmid which although reduced in size probably reduced the chance of re-infection with the parent plasmid. However plasmid survival for plasmids with more efficient conjugation systems may well be enhanced if examined in situations where cell to cell contact is more easily achieved. Also in Nature transduction has been shown to be an important process and occurred even in fresh water situations where the organisms and phage apparently manage to accumulate around surfaces or particles in relatively high numbers and, transduction may be more important than conjugation in an in vivo situation. It would be interesting to examine the behaviour of plasmids in populations maintained in an

environment providing a large surface area, for example a column containing soil or beads and possibly introducing a transducing phage.

An important factor which influences plasmid survival is the nature of the host organism (Bennet and Richmond 1978). suggested that the success of a plasmid-carrying strain in vivo depends more on the colonising properties of the organism than on the plasmid coded properties when grown in a non-selective situation. In this study and others (Melling et al. 1977, Wouters, Rops and van Andel, 1978 and Adams et al., 1979) the host organism was a laboratory maintained stock culture which had received the plasmid from a natural isolate. Costerton, Geesey and Chang (1978) have also described the differences found in laboratory grown cultures and natural isolates and demonstrated that laboratory maintained strains were at a disadvantage in vivo. The plasmid TP120 was originally isolated from Salmonella typhimurium (Grindley et al., 1973) and the effect of the plasmid on this naturally evolved plasmid-carrying strain may not be so severe since naturally isolated organisms may be more capable of "coping" and have evolved to bear the burden of the additional DNA. The plasmid RPl has been shown to affect both structure and composition of the cell envelope of E. coli (Richmond and Curtiss, 1975; Gilbert and Brown, 1978; Kenward et al., 1978) but when inserted into P. aeruginosa produced no change in cell wall phosphorus and only a slight reduction in magnesium content.

Furthermore the survival of a plasmid within a bacterial population has so far been limited to pure cultures when grown in chemostat culture. In nature, pure cultures are comparably rare events and it would be interesting to examine the survival of plasmid-carrying organisms and their interaction with other organisms in a mixed population.

While summarising some of the limitations of the chemostat culture and emphasising the difficulties in extrapolating in vitro results to a natural situation, the value of continuous culture must also be realised. When compared with the alternative approaches of batch culture growth or in vivo animal feeding experiments, the chemostat provides a more efficient and flexible tool and whilst the limitations are recognised, can produce most valuable data on plasmid behaviour.

Apart from the further research already outlined more work is also required to more fully elucidate the evolution of the plasmid TP120 and its affect on the host organism. Particularly the large increases in specific growth rates demonstrated by evolved strains is difficult to explain. As already stated it is difficult to attribute the increase in growth rate simply to a reduction of host plasmid DNA with a consequent conservation of limited growth resources (Section 7. 5). Competition studies (Section 4) have indicated that evolved strains have achieved a greater affinity for phosphorous and carbon sources which gave an

an advantage particularly at lower dilution rates. Therefore the accurate K_s values for the evolved and parent organisms should be determined. The difference in nutrient affinity might involve a change in cell membrane structure. This has already been considered in the context of the reversion of some strains, that is, E. coli K12(TP120A), E. coli K12(TP120C), E. coli K12(TP120D) and E. coli K12(TP120E) (no plasmid) to tetracycline sensitivity which is a membrane associated resistance mechanism. It would be an advantage to compare cell membranes associated with parent and evolved strains.

Lacey et al. (1975) described the evolution in vivo of penicillinase production by a plasmid in a strain of Staphylococcus aureus which was associated with a reduction in doubling time of evolved strain. This study has concentrated on the loss of recognisable markers by evolved strains and has not examined the expression or synthesis of enzymes coded for by the remaining resistance markers which may influence growth rate.

Therefore the results describe the potential for the N group plasmid to survive and evolve in unfavourable conditions. The modifications to the parent plasmid described can all be termed macroevolution (Section 7.5) (Cohen, 1978). It would be interesting to examine plasmids maintained in different host organisms by different laboratories for the evidence of macro and microevolution

,as described by Timmis et al.(1978) and Cohen et al.(1978) ,
for the plasmid R6-5 which occurred in the absence of
direct selection pressures.

The limited stability and changes in plasmid DNA
described in this thesis certainly appears to emphasise
the current view that plasmids are important vectors for
bacterial evolution. The widespread nature of the plasmids
indicate that the carriage of advantageous but non-essential
functions on extrachromosomal elements, capable of transfer
by conjugation or transduction appears to increase the
potential of individual host organisms by increasing genetic
flexibility, which proves advantageous to a bacterial
population occupying a particular environmental niche.

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G23 The Growth of Drug Resistant *Escherichia coli* in Continuous Culture in the Absence of Drugs. Dianne Godwin and J.H. Slater (Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL).

A strain of *Escherichia coli* K12 containing the autotransferable plasmid TP120 of compatibility group N with resistance to ampicillin (Ap), streptomycin (Sm), sulphonamide (Su) and tetracycline (Tc) was grown under either carbon- or phosphorus-limited conditions at a variety of dilution rates in a defined medium lacking any of the drugs. In glucose-limited chemostats resistance to Tc was routinely lost resulting in the competitive takeover of a second population sensitive to Tc but retaining resistance to Ap, Sm and Su. Prolonged continuous growth, in some cases for a further 2000 h, failed to cause any further loss of the remaining drug resistances. One strain carrying resistance to Ap, Sm and Su isolated from a selection experiment at $D = 0.1 \text{ h}^{-1}$ was shown to contain a transferable plasmid. In contrast under phosphate-limited conditions one or more of the other drug resistance markers were also lost resulting in the selection of a number of different dominant populations with varying plasmid genotypes depending on each selection experiment. Several strains carrying resistance to different drug combinations were shown to contain plasmids although in two cases there was an apparent loss of the transfer capacity. In a single selection experiment a plasmid-minus strain sensitive to all four drugs was isolated. In all the selection experiments undertaken very low levels of the uncompetitive, replaced parent populations were maintained in the cultures.

Batch culture experiments showed that the original parent strain, resistant to Ap, Sm, Su and Tc, had a maximum specific growth rate, μ_{max} , of 0.40 h^{-1} . Various two and three drug resistant isolates showed μ_{max} values approximately 30% greater than the parent whilst the plasmid-minus strain was capable of twice the parent growth rate. Continuous-flow culture competition experiments between the parent four drug resistant strain and a strain having lost resistance to tetracycline showed that over the dilution rate range $D = 0.1$ to 0.35 h^{-1} , the tetracycline sensitive organism was always the most competitive, an advantage which was greater at low dilution rates.

The Influence of the Growth Environment on the Stability of a Drug Resistance Plasmid in *Escherichia coli* K12

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(Received 25 September 1978)

Populations of *Escherichia coli* K12 containing the plasmid TP120 which coded for resistance to ampicillin, streptomycin, sulphonamide and tetracycline were grown in a chemostat under carbon-limited and phosphorus-limited conditions. With time, resistance to one or more of the drugs was lost, resulting in the production of mutant populations which were more competitive than the parent population. The resistance to tetracycline was always lost under both carbon and phosphorus limitations, but resistance to the other three drugs was lost only during phosphate-limited growth. Strains of *E. coli* which had lost resistance to one or more of the drugs were capable of higher maximum specific growth rates than the parent strain.

INTRODUCTION

Plasmids coding for drug resistances have been extensively investigated in terms of their biochemical, genetic and epidemiological properties but relatively little is known about the behaviour of plasmids and plasmid-carrying organisms under different environmental and physiological conditions. In particular, the effect of different growth environments and host growth rates on the survival and stability of extrachromosomal elements within a population has only recently been considered (Melling *et al.*, 1977). Experiments with R factor-carrying *Escherichia coli* in the human gut have shown that the antibiotic-resistant population disappeared rapidly which suggested that the plasmid-containing population was less competitive than the resident populations (Anderson, 1974; Anderson, E. S., 1975). Fresh challenges with antibiotics resulted in the re-establishment of the resistant population (Anderson *et al.*, 1973). Under non-challenge conditions it was suggested that the loss of the resistant population might be due to impaired growth rates of the plasmid-containing population compared with the plasmid-minus population (Anderson, 1974) and/or inhibition of plasmid transfer by bile salts, inert bacterial material and the density of the competitive organisms (Anderson, J. D., 1975). Conversely, in water supplies and raw sewage, drug-resistant coliforms showed a long-term survival and capacity to transfer the resistance factors in mixed populations (Grabow *et al.*, 1974). Antibiotic-resistant Enterobacteriaceae occur at high frequencies in aquatic environments and it has been suggested that they survive well under these conditions (Smith, 1970; Freary *et al.*, 1972; Hughes & Meynell, 1975).

A number of closed culture studies have all shown that plasmid-containing microbial populations had lower maximum specific growth rates than plasmid-minus populations (Terawaki *et al.*, 1968; Yokota *et al.*, 1972; Grinstead & Lacey, 1973; Lacey, 1975; Kayama & Yara, 1975; Inselburg, 1978; Nakazawa, 1978). This strongly suggests that plasmid-containing populations are at a competitive disadvantage under growth conditions where the characteristics coded for by the plasmid are not or do not need to be expressed. Melling *et al.* (1977), using chemostat continuous-flow culture techniques, showed that *E. coli*

This study was undertaken to investigate the effects of various growth conditions (carbon and phosphorus limitation) and growth rates, using continuous-flow culture techniques, on the stability of a plasmid coding for resistance to ampicillin, streptomycin, sulphonamide and tetracycline in *E. coli* K12 in the absence of any of the drugs.

METHODS

Two-membered mixed culture competition experiments were performed with *E. coli* K12(TP120) and *E. coli* K12(TP120A). Separate overnight cultures grown in glucose mineral salts medium were simultaneously inoculated into a glucose-limited chemostat to give approximately equal population densities. After a period of closed culture growth, to overcome problems associated with variable lag phases and to ensure that both populations were growing exponentially, the fresh medium flow was initiated to give the selected dilution rate, ranging from 0.10 to 0.35 h⁻¹. Samples were taken at 3 to 4 h intervals for viable organism determination by plating on nutrient agar and nutrient agar supplemented with tetracycline at 25 µg ml⁻¹. Viable counts were determined after 24 h at 37 °C. The specific growth rate of the uncompetitive population, μ_u , was calculated in two ways. Firstly, using a modified washout growth rate equation due to Jannasch (1969):

growth conditions (carbon
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ptomycin, sulphonamide

where R_0 is the initial ratio of x_1 and μ_2 is the specific growth rate of the culture $\mu_2 = D$ (S

Conjugation. T obtained by conjugating plasmid-minus *E. coli* broth to the end grown to mid-exponential phase (2×10^8 organisms) with $10 \mu\text{g}$ ampicillin to 10^{-8} dilution with *E. coli* NaI suitable dilution had received by and the frequency. For each conjugation supplement resistance marker

[illegible]

$$\mu_1 = \frac{\ln x_1 - \ln x_0}{(t_1 - t_0)} + D \quad (1)$$

where x_0 is the initial uncompetitive population size at time t_0 , x_1 is the final uncompetitive population size at time t_1 and D is the dilution rate maintained for the duration of the experiment. Secondly, the difference in the specific growth rates of the two competing populations was calculated from:

$$(\mu_1 - \mu_2) = \frac{\ln R_1 - \ln R_0}{(t_1 - t_0)} \quad (2)$$

where R_0 is the initial ratio of the competitive population to the uncompetitive population at time t_0 , R_1 is the final ratio of the two populations at time t_1 , μ_1 is the specific growth rate of the uncompetitive population x_1 and μ_2 is the specific growth rate of the competitive population x_2 . When $x_2 \gg x_1$, then in chemostat culture $\mu_2 = D$ (Slater & Bull, 1978) and so:

$$\mu_1 = \frac{\ln R_1 - \ln R_0}{(t_1 - t_0)} + D \quad (3)$$

Isolation of plasmid DNA. Plasmid DNA was isolated by a dye buoyant caesium chloride density gradient centrifugation method (Palchaudhuri & Chakrabarty, 1976) as modified by K. G. Hardy (University of Kent, personal communication).

Conjugation. The maintenance, or otherwise, of an autotransferable plasmid in the various isolates obtained by continuous-flow culture selection was demonstrated by showing its potential to transfer to a plasmid-minus recipient (Dennison & Baumberg, 1975). Cultures of the recipient were grown in nutrient broth to the end of the exponential phase giving approximately 5×10^8 organisms ml^{-1} ; the donors were grown to mid-exponential phase in either nutrient broth or the minimal media, giving approximately 2×10^8 organisms ml^{-1} . Recipient *E. coli* Nal^R (0.1 ml) was spread on nutrient agar plates supplemented with 10 μg ampicillin ml^{-1} or 10 μg sulphonamide ml^{-1} . The donor strains were serially diluted separately to 10^{-4} dilution and 0.02 ml of each dilution was inoculated dropwise on to the surface of the plates spread with *E. coli* Nal^R. The plates were incubated for 18 h at 37 °C and the number of colonies growing at a suitable dilution were counted. The colonies represented nalidixic acid-resistant *E. coli* organisms which had received by conjugation a plasmid carrying ampicillin or sulphonamide resistance (transconjugants), and the frequency of transfer was expressed as the percentage of transconjugants to total donor organisms. For each conjugation experiment, a number of transconjugant colonies were transferred to a range of media supplemented separately with the four drugs to check for the concomitant transfer of all the expected resistance markers.

RESULTS

Plasmid stability and strain selection

The stability of populations of *E. coli* containing plasmid TP120 was examined under glucose-limited and phosphate-limited conditions at dilution rates between 0.10 and 0.35 h^{-1} . In all experiments, at least one of the drug resistance markers was eventually eliminated from the population. However, the pattern of loss of markers from glucose-limited cultures was different from that from phosphorus-limited cultures. In all the glucose-limited cultures only the resistance to tetracycline was lost. In the experiment shown in Fig. 1, the parent population, as measured by the loss of tetracycline resistance, declined by a factor of more than 10^4 between 500 and 800 h after changing to drug-free medium; it was replaced by a second population, *E. coli* K12(TP120A), lacking resistance to tetracycline but retaining resistance to ampicillin, streptomycin and sulphonamide. For a further 1000 h of growth there was continuous presence of the parent population at concentrations varying between 10 and 10^3 organisms ml^{-1} (up to 0.0001 % of the total population). During this time no additional changes in the dominant population were apparent and there was no further loss of drug resistance markers. The dominant population from the selection experiment at $D = 0.10 \text{ h}^{-1}$, *E. coli* K12(TP120A), contained a plasmid, as shown by density gradient centrifugation following cell lysis, and retained the capacity to transfer a plasmid carrying resistance to ampicillin, streptomycin and sulphonamide (Table 1). The other strains showing similar phenotypes to *E. coli* K12(TP120A) that were isolated from selection experiments at higher dilution rates were not analysed further.

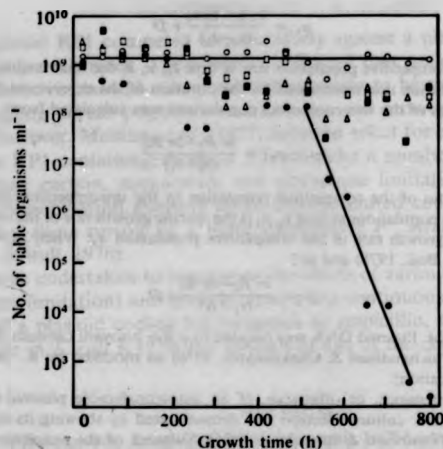


Fig. 1. The growth of *E. coli* K12(TP120) in a glucose-limited chemostat at a dilution rate of 0.10 h^{-1} illustrating the displacement of the tetracycline resistance marker (and hence the parent population) and the domination of a derivative strain [*E. coli* K12(TP120A)] resistant to ampicillin, streptomycin and sulphonamide but sensitive to tetracycline. Time 0 is the time at which drug-free medium flow was started. Total number of viable organisms (○), and number of viable organisms resistant to tetracycline (●), ampicillin (□), streptomycin (■) and sulphonamide (△).

Table 1. Presence of plasmids in selected *E. coli* K12 strains and frequencies of plasmid transfer

The presence of plasmids was examined by density gradient centrifugation. Plasmid transfer ability was tested with the plasmid-minus recipient *E. coli* K12 Na¹⁸. Strains were derived from continuous-flow selection experiments at a dilution rate of 0.10 h^{-1} under carbon-limited conditions (a) or phosphorus-limited conditions (b).

<i>E. coli</i> K12 with plasmid (resistance markers)	Presence of plasmid	10 ⁴ × Percentage frequency of transfer of markers		
		Nutrient broth	Davis & Mingioli defined medium	Hershey defined medium
TP120 (Ap, Sm, Su, Tc)	+	5.5	5.3	1.8
TP120A ^a (Ap, Sm, Su)	+	5.0	0.1	0.3
TP120B ^b (Sm, Su, Tc)	+	1.4	0.1	0.6
TP120C ^a (Ap, Sm, Su)	+	0	0	0
TP120D ^b (Sm, Su)	+	0	0	0

A more complex pattern of loss of drug resistance markers associated with an increase in parent strain instability was observed in the phosphate-limited selection experiments. In common with selection under carbon-limited conditions, the tetracycline marker was routinely lost from steady state cultures at similar washout rates, but it was never completely eliminated and replaced by a more competitive tetracycline-minus mutant as might be expected theoretically. In only one instance under phosphate-limited conditions at

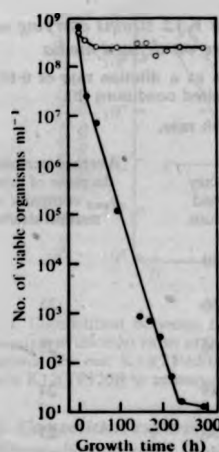


Fig. 2

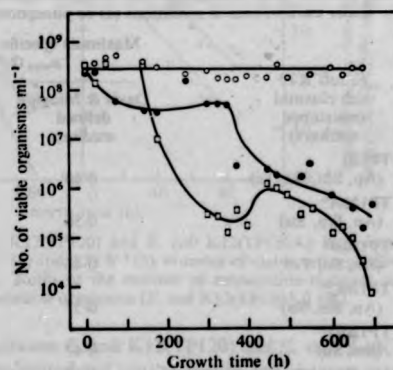


Fig. 3

Fig. 2. The growth of *E. coli* K12(TP120) in a phosphate-limited chemostat at a dilution rate of 0.10 h^{-1} showing the loss of the tetracycline resistance marker and hence the parent population. The replacement population [*E. coli* K12(TP120C)] was resistant to ampicillin, streptomycin and sulphonamide, and all three resistances were individually present at above 10^8 resistant organisms ml^{-1} for the duration of the experiment. Time 0 is the time at which drug-free medium flow was started. Total number of viable organisms (\circ), and number of viable organisms resistant to tetracycline (\bullet).

Fig. 3. The growth of *E. coli* K12(TP120) in a phosphate-limited chemostat at a dilution rate of 0.35 h^{-1} showing the loss of ampicillin and tetracycline resistance markers. Time 0 is the time at which drug-free medium flow was started. Total number of viable organisms (\circ), and number of viable organisms resistant to tetracycline (\bullet) and ampicillin (\square).

$D = 0.10 \text{ h}^{-1}$ was tetracycline resistance the sole marker lost (Fig. 2). In this experiment the original population resistant to the four drugs persisted at a level of approximately 10 organisms ml^{-1} in a total population of 4×10^8 *E. coli* K12(TP120C) organisms ml^{-1} . Throughout the experiment all the other three drug resistances were individually shown to be present at concentrations above 10^8 resistant organisms ml^{-1} demonstrating that the disappearance of tetracycline resistance reflected only the washout of the parent population. Unlike the loss of tetracycline-resistant organisms under glucose-limited conditions, however, with phosphate as the growth-limiting nutrient the disappearance of the tetracycline resistance marker usually began soon after the removal of the drugs from the growth environment (Fig. 2), although this was not always the case (Fig. 3). A number of different selection experiments yielded dominant derivative strains with the same phenotype as *E. coli* K12(TP120C), but only the strain isolated at $D = 0.10 \text{ h}^{-1}$ was analysed and shown to contain a plasmid (Table 1). Unlike the comparable strain isolated from carbon-induced selection [*E. coli* K12(TP120A)], numerous attempts failed to demonstrate any retention of the capacity to transfer the derived plasmid into the recipient strain (Table 1).

An important difference between carbon-limited and phosphorus-limited stability and selection experiments (with the single exception of the phosphate-limited selection experiment just mentioned) was that under phosphate-limited conditions drug resistance markers other than tetracycline were also lost which led to the selection of different dominant populations with various plasmid genotypes in different experiments. Furthermore, these experiments produced mixed cultures with two or more different strains containing various

at a dilution rate of (and hence the parent A)] resistant to ampicillin is the time at which and number of viable sulphonamide (Δ).

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ion. Plasmid transfer ins were derived from r carbon-limited con-

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ciated with an increase selection experiments, tracycline marker was but it was never com- minus mutant as might -limited conditions at

Table 2. Maximum specific growth rates for selected *E. coli* K12 strains carrying various drug resistance markers (or none) in closed culture on defined media

Strains were derived from continuous-flow selection experiments at a dilution rate of 0.10 h⁻¹ under carbon-limited conditions (a) or phosphorus-limited conditions (b).

<i>E. coli</i> K12 with plasmid (resistance markers)	Maximum specific growth rate, μ_{max} (h ⁻¹)		Average percentage decrease of parent μ_{max} compared with mutant strains
	Davis & Mingioli defined medium	Hershey defined medium	
TP120 (Ap, Sm, Su, Tc)	0.40	0.39	—
TP120A ^a (Ap, Sm, Su)	0.56	0.50	25
TP120B ^b (Sm, Su, Tc)	0.46	0.60	25
TP120C ^b (Ap, Sm, Su)	0.57	0.48	24
TP120D ^b (Sm, Su)	0.47	0.56	23
No plasmid ^b	0.69	0.90	50

combinations of drug resistance markers. For example, in one selection at $D = 0.10$ h⁻¹, after 300 h growth in drug-free medium the culture was dominated by *E. coli* K12(TP120D) which carried resistance only to sulphonamide and streptomycin and contained a non-transferable plasmid (Table 1). After this period of growth the culture also contained a population only lacking resistance to tetracycline [that is, showing the same phenotype as *E. coli* K12(TP120A) and K12(TP120C)] at 0.001% of the total population and a second population lacking resistance to ampicillin at 1% of the total population. The latter strain was isolated, designated *E. coli* K12(TP120B) and shown to carry resistance to streptomycin, sulphonamide and tetracycline and to contain a transferable plasmid (Table 1).

Another feature of populations growing with phosphate as the limiting nutrient was that the washout curves for the loss of individual resistance markers rarely followed the anticipated washout kinetics (Fig. 3). The change in the logarithm of the population(s) with resistance to a specific drug was usually non-linear, exhibited plateau periods and occasionally short phases of transient increase in drug resistance before washout continued (Fig. 3).

Competition between the parent organism, E. coli K12(TP120) (Ap, Sm, Su, Tc), and a tetracycline-sensitive organism, E. coli K12(TP120A) (Ap, Sm, Su), in open culture

The continuous-flow culture stability experiments showed that, particularly under phosphate-limited growth conditions, it was possible to select for mutant strains carrying decreased numbers of drug resistance markers. Indeed, the final stage of one phosphate-limited stability experiment yielded an organism lacking resistance to all four of the drugs and which could not be shown to contain a plasmid. Furthermore, in prolonged selection experiments, the derivative strains that were resistant to three or less of the drugs tended to dominate the original organism suggesting that under the prevailing growth conditions these strains had a selective growth advantage. Closed culture experiments (Table 2) showed that the four mutant strains carrying plasmids coding for resistance to two or three drugs were capable of faster growth rates with maximum specific growth rates (μ_{max}) approximately 30% greater than the parent strain. This trend was continued by the plasmid-minus strain whose μ_{max} value was greater than those of the plasmid-containing mutants and twice the μ_{max} of *E. coli* K12(TP120). However, these differences may not be

Table 3. Maximum specific growth rates for selected *E. coli* K12 strains carrying various drug resistance markers (or none) in closed culture on defined media

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No plasmid ^b	0.69	0.90	50

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Fig. 4. Competition curves for *E. coli* K12(TP120) and *E. coli* K12(TP120A) in open culture.Table 3. Comparison of growth rates of *E. coli* K12(TP120) and *E. coli* K12(TP120A) in open culture.

Nutrient
limitation
Carbon
(glucose)

Phosphorus
(phosphate)

significant differences in growth rates. For example, in one selection at $D = 0.10$ h⁻¹, after 300 h growth in drug-free medium the culture was dominated by *E. coli* K12(TP120D) which carried resistance only to sulphonamide and streptomycin and contained a non-transferable plasmid (Table 1). After this period of growth the culture also contained a population only lacking resistance to tetracycline [that is, showing the same phenotype as *E. coli* K12(TP120A) and K12(TP120C)] at 0.001% of the total population and a second population lacking resistance to ampicillin at 1% of the total population. The latter strain was isolated, designated *E. coli* K12(TP120B) and shown to carry resistance to streptomycin, sulphonamide and tetracycline and to contain a transferable plasmid (Table 1).

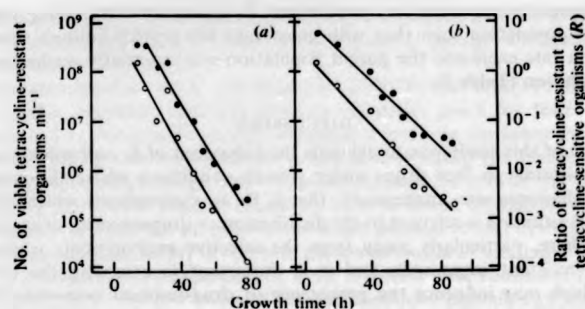


Fig. 4. Competition between *E. coli* K12(TP120) and *E. coli* K12(TP120A) in glucose-limited chemostats at dilution rates of 0.10 h⁻¹ (a) and 0.35 h⁻¹ (b). Number of viable tetracycline-resistant organisms [*E. coli* K12(TP120)] (○). Ratio of the number of tetracycline-resistant organisms [*E. coli* K12(TP120)] to tetracycline-sensitive organisms [*E. coli* K12(TP120A)] (●).

Table 3. Competition experiments between *E. coli* K12(TP120) and *E. coli* K12(TP120A) at different dilution rates in carbon-limited and phosphorus-limited chemostat cultures

Nutrient limitation	Dilution rate (h ⁻¹)	Specific growth rate, μ , of uncompetitive population [<i>E. coli</i> K12(TP120)]		Average percentage decrease of μ for uncompetitive population [<i>E. coli</i> K12(TP120)]
		From eqn 1 (Jannasch, 1969)	From eqn 3 (Slater & Bull, 1978)	
Carbon (glucose)	0.10	-0.02 (0)	-0.03 (0)	100
	0.15	0.05	0.05	67
	0.25	0.17	0.17	32
	0.35	0.27	0.26	24
Phosphorus (phosphate)	0.10	0.01	0.02	85
	0.15	0.03	0.04	77
	0.25	0.16	0.16	36
	0.35	0.23	0.21	37

significant during growth under substrate-limited conditions and submaximal specific growth rates. A detailed examination of the kinetics of competition between one pair of organisms differing in sensitivity towards tetracycline, namely *E. coli* K12(TP120) and *E. coli* K12(TP120A), showed that under both carbon-limiting and phosphorus-limiting conditions, and at all the growth rates examined, the tetracycline-sensitive population always had a competitive growth advantage over the tetracycline-resistant population (Fig. 4; Table 3). In these experiments, care was taken to ensure that both the newly inoculated populations were growing exponentially before establishing continuous growth conditions. Thus, differences in the growth dynamics of the competing populations were ascribable to differences in growth parameters and were not obscured by additional factors such as variable lag phases or differences in physiological status. For all competition experiments, the removal of the uncompetitive tetracycline-resistant *E. coli* K12(TP120) followed the expected washout kinetics (Fig. 4). At the highest dilution rate examined ($D = 0.35$ h⁻¹) for carbon-limited competition, the percentage decrease in the specific growth rate of the uncompetitive parent population was similar to the difference between the maximum specific growth rates in closed culture (Tables 2 and 3). At the same dilution rate with phosphate as the growth-limiting substrate, the parent strain was even more uncompetitive since, with existing growth conditions determined by *E. coli* K12(TP120A), *E. coli* K12(TP120) was only capable of a growth rate of 0.22 h⁻¹ (Table 3). The degree

of competitiveness between the two populations increased with decreasing growth rate of the successful population such that with glucose as the growth-limiting substrate at the lowest dilution rate examined the parent population was essentially washed out as a non-growing population (Table 3).

DISCUSSION

The purpose of this study was to examine the behaviour of *E. coli* containing a plasmid coding for resistance to four drugs under growth conditions where the presence of the resistance mechanisms was unnecessary, that is in an environment which lacked any of the drugs. This situation is relevant to the distribution of drug-resistant organisms throughout the biosphere, particularly away from the selective environments which led to the evolution of resistant organisms, and it is important in assessing the environmental conditions which may influence the persistence of drug-resistant organisms and plasmid stability. In drug-free environments, the plasmid is redundant and environmental pressures could then discriminate against plasmid-containing organisms, since synthesis and replication of the plasmid utilizes elemental and energy resources which otherwise might be diverted to biomass production and a high population growth rate. This principle was demonstrated by Zamenhof & Eichhorn (1967) who showed that amino acid auxotrophs of *Bacillus subtilis* had a marked growth rate advantage over isogenic prototrophic strains under carbon-limited growth conditions. Furthermore, the greater the number of biosynthetic sequences dispensed with by the auxotroph the greater was its growth rate advantage over the prototroph. Conversely organisms which wasted growth nutrients, for example, by overproducing and excreting an amino acid, were at a competitive disadvantage (Zamenhof & Eichhorn, 1967; Baich & Johnson, 1968).

In view of the strongly selective growth conditions occurring in chemostat continuous-flow culture (Cox & Gibson, 1974; Harder *et al.*, 1977; Slater & Bull, 1978) and the fact that there was a large difference between the specific growth rates of plasmid-containing and plasmid-minus strains of *E. coli* (Table 2), it was anticipated that mutant plasmid-minus strains would have been rapidly selected for under nutrient-limited growth conditions. Our experiments showed that this was not the case and, indeed, in only one instance – under phosphate-limited conditions after approximately 700 h growth – was a plasmid-minus population detected. Thus the plasmid of *E. coli* K12(TP120) appeared to be considerably more stable than the *F'lac* plasmid in *E. coli* under lactose-limited growth conditions (Slater & Bull, 1978). However, unlike the RP1 plasmid in *E. coli* strain W3110, which was stably maintained for up to 144 h growth under conditions of carbon, phosphorus or magnesium limitation at growth rates between 0.05 and 1.0 h⁻¹ (Melling *et al.*, 1977), plasmid TP120 was only partially stable. Particularly under phosphate-limited conditions, the cultures readily lost resistance to one or two drugs which suggested that the plasmid could partially fragment, perhaps producing a smaller plasmid, and hence gain an advantage in growth rate over the parent strain. Large multiple resistance plasmids seem to be formed as the result of the linkage of separately derived resistance genes or groups of genes to a basic replicating and transferring unit (RTF replicon) and this is a reversible process (Cohen, 1976). Clearly, this provides a mechanism for the generation of smaller plasmids by random reassociation without one (or more) of the original DNA segments and preliminary evidence suggests that this was the case here. The fact that tetracycline resistance was regularly lost indicated that this particular sequence was more readily excised from the original plasmid than the other resistance genes. At present we can offer no satisfactory explanation of the observation that the loss of drug resistances occurred more readily under phosphate-limited conditions than under carbon-limited conditions, particularly as the competition experiments between strains K12(TP120) and K12(TP120A) showed a greater growth rate difference under carbon limitation than under phosphorus limitation (Table 3). The loss of the autotransferring capacity in strains

decreasing growth rate of limiting substrate at the fully washed out as a non-

coli containing a plasmid where the presence of the mutant which lacked any of the resistant organisms through- elements which led to the environmental pressures and plasmid environmental pressures since synthesis and repli- which otherwise might be rate. This principle was amino acid auxotrophs of isogenic prototrophic strains water the number of bio- was its growth rate tested growth nutrients, for competitive disadvantage

in chemostat continuous- & Bull, 1978) and the fact rates of plasmid-containing that mutant plasmid- limited growth conditions. ed, in only one instance – growth – was a plasmid- 2(TP120) appeared to be under lactose-limited growth id in *E. coli* strain W3110, conditions of carbon, phos- and 1.0 h⁻¹ (Melling *et al.*, drugs which suggested that smaller plasmid, and hence multiple resistance plasmids derived resistance genes or RTF replicon) and this is a mechanism for the generation (more) of the original DNA the case here. The fact that particular sequence was more ance genes. At present we the loss of drug resistances than under carbon-limited en strains K12(TP120) and carbon limitation than under transferring capacity in strains

K12(TP120C) and advantage either the non-expressio

The competitio putatively smaller mutant strain had of the growth-lin between the muta gained in losing removal of part c in the specific gro

Finally, in all of continuous gr were maintained. (1977), although several orders of not obvious sin populations oug system artifacts homogeneous cc excluded that so advantageous ca currently the p population subj of the populatio

D.G. was sup We are grateful and G. Willsha assistance.

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K12(TP120C) and K12(TP120D) also appeared to contribute towards a selective growth advantage either through the loss of the transfer genes or through a mutation resulting in the non-expression of the genes.

The competition studies with *E. coli* strains K12(TP120) and K12(TP120A) showed that putatively smaller plasmids, certainly carrying resistance genes for fewer drugs, in the mutant strain had a considerable growth advantage. Moreover, the lower the concentration of the growth-limiting substrate (that is, at low growth rates) the greater the difference between the mutant and parent growth rate potentials and the greater the advantage to be gained in losing part of the plasmid (Table 3). It is, however, difficult to explain why the removal of part of the plasmid or, indeed, the whole plasmid results in such large increases in the specific growth rate simply in terms of the conservation of limiting growth resources.

Finally, in all the selection experiments, some of which were continued for over 2000 h of continuous growth, very low levels of the uncompetitive plasmid-containing organisms were maintained. This is an observation in agreement with the experiments of Melling *et al.* (1977), although as a percentage of the population our residual resistant populations were several orders of magnitude lower. The reason for the persistence of these populations is not obvious since kinetic theory predicts that in open growth systems uncompetitive populations ought to be completely eliminated. Most probably these low levels are growth system artifacts representing a small population retained as a result, for example, of non-homogeneous conditions within the culture vessel or wall growth. However, it cannot be excluded that some mechanism, so far unidentified, exists whereby low levels of a potentially advantageous capability (drug resistance) are retained within the population even though currently the population may be disadvantaged. Clearly, the long-term survival of the population subjected to future drug challenges would be greatly aided if a few members of the population retained the necessary resistance mechanisms.

D.G. was supported by a Research Studentship from the Medical Research Council. We are grateful to Professor D. C. Ellwood and Drs K. P. Flint, K. G. Hardy, H. Smith and G. Willshaw for helpful discussions and Mrs D. E. Sanders for valuable technical assistance.

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